coup de grace of myocardial infarction no one would deny its supreme importance

If the participants can clarify the field by dropping the white of an egg into it they will have performed a signal service

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The Role of the Connective Tissues in Arterial Diseases

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It seems unfortunate that in recent years so much attention has been paid to the fatty changes in arterial disease and so little to the processes responsible for arterial thickening Admittedly fatty change may account for some of the thickening in atherosclerosis but by far the more important factor is the increase in fibrous tissue which causes the intima to encroach upon the lumen of the artery and narrow it When we consider all the lesions ascribed to impairment of the circulation from narrowing of the arteries we are forced to regard this as one of the most important processes in human pathology

In the past we have taken it for granted that arterial thickening represents a change in the arterial connective tissues either a reactive overgrowth as in atherosclerosis or a degenerative swelling as seen in the hyaline change of arterioles in hypertension These assumptions are based on histology and provide a reasonable explanation of the histological picture but when we take the functional activity of arteries into account they lead us into difficulties

Arteries are elastic tubes supporting a high internal pressure of blood and we would therefore expect them to give way and dilate when they degenerate rather than become narrowed It is true that a fibrous overgrowth might strengthen an artery and possibly help it to resist dilatation but it would not account for narrowing The larger arteries depend on their elasticity to withstand the distending forces of the blood pressure With each pulse wave they dilate and recoil again and while the dilatation is a movement imposed on them by the pulse pressure the recoil is a function of their own elasticity Therefore anything like a fibrous overgrowth which interferes with their elasticity will tend to leave them dilated yet in atherosclerosis we sometimes find them narrowed almost to the point of occlusion

This is a problem which exercised many pathologists in the last century and it was almost by accident that a solution presented itself to me I was demonstrating the principles of organization to a class of students using a thrombosed and canalized coronary artery (Fig 1) Each member of the class had his own section and in the course of the

demonstration I noticed some were in difficulties because instead of a canalized artery they had an artery with a thickened intima (Fig 2) It was obviously the same vessel but in cutting the sections the technician had gone beyond the occluded part This fortunate accident drew our attention to an aspect of arterial thrombosis which held the key to the problem When the sections were recalled and examined in serial order the fibrous mass which represented the occluding thrombus in Fig 1 was found to be continuous with that which formed the intimal thickening in Fig 2 In other words the thrombus did not end with the occlusion but extended further along the artery as a fibrous thickening

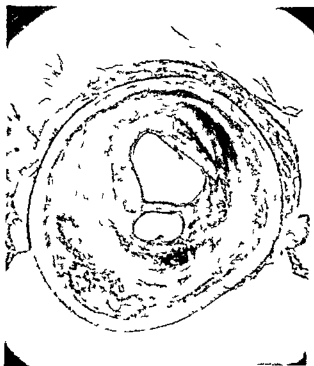


FIG 1 Magnification × 18

Here was evidence that thrombosis could be a source of arterial thickening and in fact the solution to the problem of narrowing A thrombus occupying the lumen of an artery was bound to narrow it and the only difficulty now was to understand how thrombosis could fit into the picture of *atherosclerosis*

Fifteen years ago arterial thrombosis was thought to be an uncommon occurrence whereas atherosclerosis was universal so that the pros

Fig 2. Magnification × 18



Fig 3 Magnification × 15



Fig 4 Magnification × 122

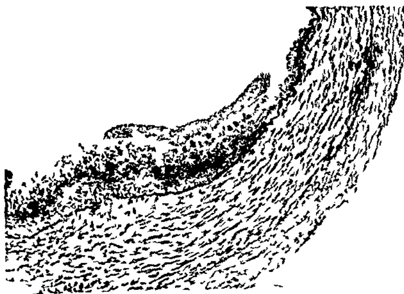


Fig 5 Magnification × 85

pect of showing that the two were essentially related seemed remote Nevertheless when I set out to search for thrombi in the aorta and coronary arteries I found they were remarkably common Thrombi of all kinds were to be found ranging from large masses almost filling the lumina of the arteries down to mural deposits (Fig 3) some of them consisting of thin encrustations of fibrin (Fig 4) which were hardly detectable Some were recent (Fig 5) and others of long standing and partly organized (Fig 6) and from a study of the whole range the following new principle came to light When a mural thrombus forms in an artery it becomes covered with endothelium so that it is incorporated in the vessel wall with the result that on subsequently being organized it forms a fibrous thickening of the intima



Fig 6 Magnification × 85

The acceptance of this principle is I believe essential for the understanding of arterial pathology but some observers will hesitate to accept it on purely histological grounds The experiments carried out by Dr Lichtenberger in my department although they do not actually involve thrombosis prove a useful confirmation because they show the same essential process in operation Lichtenberger using a method previously described by Harrison (1) and Heard (2) injected fine particles of autogenous fibrin into the ear veins of rabbits so as to produce emboli

in the pulmonary arteries Figure 7 shows an embolus after 48 hours already attached to the vessel wall and covered with endothelium

Figure 8 shows another after 4 days now covered by a double layer of cells and more or less molded to the shape of the vessel wall

Figure 9 shows an embolus after 2 months now incorporated in the wall and forming what might be taken for a swelling or overgrowth of the intimal tissues



FIG 7 Magnification × 60

Finally Fig 10 shows an affected artery after 8 months with thickenings in its wall but little or nothing in their appearance to indicate they originated as emboli

This phenomenon explains many hitherto obscure features of atherosclerosis Mural thrombi before being organized pass through a succession of changes which tend to obscure their identity and mislead the histologist Since the process frequently recurs with one deposit forming on top of another different stages can sometimes be seen in one section (Fig 11) Recent thrombi have the loose fibrillary or sometimes granular



Fig 8 Magnification × 120



Fig 9 Magnification × 100

structure characteristic of fibrin and, since they usually stain specifically are easily identified On the other hand older thrombi under the influence of the blood pressure become condensed and hyaline and sometimes laminated and lose their specific staining properties so that they are easily mistaken I think there is little doubt that some changes referred to as 'hyaline or fibrinoid degeneration in atherosclerosis



Fig 10 Magnification × 120



Fig 11 Magnification × 40

have actually been altered fibrin thrombi In the end the fibrin is usually converted into fibrous tissue Thus an artery with successive deposits on its intimal surface becomes increasingly thickened with complex strata of mixed hyaline and fibrous tissue (*Fig* 12) and when fatty deposits are superadded the picture of atherosclerosis is completed

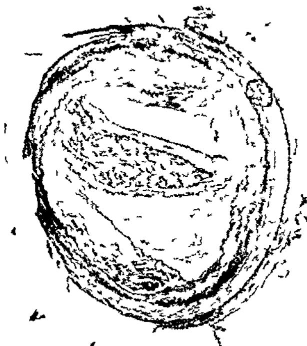


FIG 12 Magnification × 35

As I have explained the orthodox overgrowth idea is difficult to reconcile with functional considerations but it has long prevailed for the simple reason that the intimal thickenings *look like* overgrowths We now know however from the experiments cited above that appearances are deceptive and that tissues which appear to be part of a vessel may actually be derived from the blood It is not my purpose here to push any new theory of atherosclerosis or to argue that all intimal thickenings are products of thrombosis But I would emphasize that those who are concerned with the study of connective tissues in arteries should be aware of these considerations and should be cautious in the identification of the various components of arterial lesions

The same argument applies to all arteries large or small Any degeneration of their walls must reduce their efficiency or in other words their ability to withstand the distending forces of the blood pressure We should therefore expect degenerate arteries to be dilated yet in hyaline arteriolosclerosis of hypertension we see what is commonly regarded as a degeneration associated with narrowing and naturally my experience with the larger arteries led me to wonder if something like a surface deposit might be involved in them also

In the typical hyaline arteriolosclerosis there is usually a relatively thick layer of homogeneous material surrounding the lumen and constricting it

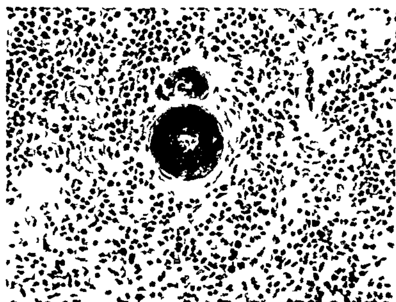


Fig 13 Magnification × 370

As a rule the material stains a bright pink with eosin and is PAS positive but otherwise stains indifferently so that its nature is uncertain Although the majority of affected arterioles have a prominent endothelial lining the hyaline material may possibly originate as a surface deposit and be covered later by endothelium If such were the case however it should sometimes be possible to see a deposit before it has been covered and with this in mind I searched through *sections of kidney and spleen from a series of cases of hypertension* and diabetes I found that the evidence in support of the deposition hypothesis was even more favorable than I expected

I should make it clear that the examples illustrated here are com

paratively rare findings Nearly all the affected arterioles showed the usual appearance but here and there an occasional one was found in which the endothelial lining was missing (Figs 14 and 15) and the hyaline material exposed to the blood stream In some instances it was actually mixed in loose floccules with the blood (Fig 16) while in some the arterioles were choked with hyaline material (Fig 17) Moreover the material in the blood corresponded in all respects with that in the walls and seemed to be identical It was usually negative to fibrin stains except in the cases of malignant hypertension where the arterioles had



Fig 14 Magnification × 470

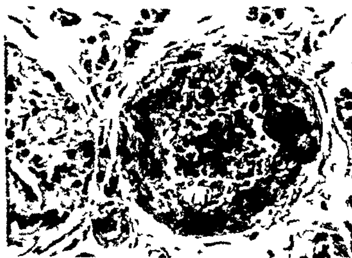


Fig 15 Magnification × 470

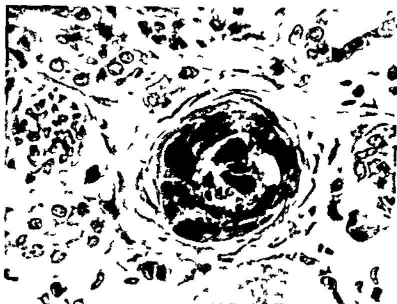


Fig 16 Magnification × 470



Fig 17 Magnification × 470

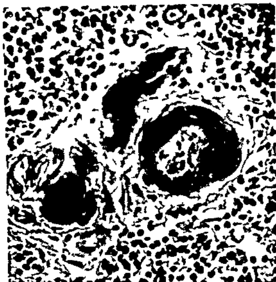


FIG 18 Magnification × 370

the appearance referred to as fibrinoid necrosis (Fig 18) Thus the hyaline material may sometimes be fibrin in an altered state or possibly a coagulum formed of some other elements of the circulating blood

These pictures as I have said are rare examples and I do not present them as proof that hyaline change represents a surface deposition but I think the fact that they occur and conform so closely to what would be expected of surface depositions is significant I believe that in the arterioles as in the larger arteries there is a process by which solid matter is eliminated from the blood stream by being incorporated in the vessel walls Whether or not it is responsible for hyaline arteriolo sclerosis I leave others to decide but I would emphasize that in con sidering questions of connective tissue changes in arterioles that possi bility should be borne in mind

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DISCUSSION

DR HARTROFT We too have recently been studying plaques in aortas You said you used frozen sections to prepare several that showed recently organized thrombi Do you think very small ones that are probably only 6 or 7 micra thick are missed in paraffin sections because of shrinkage or contraction? We have seen exactly what you have shown of recently organized thrombi in aortas in doing fat stains on frozen sections and yet we do not see them as well in paraffin sections

I suspect this stage of the lesion may have been missed frequently because of shrinkage in paraffin sections which plaster the plaques against the wall and render them inconspicuous

Dr Duguid I am sure you are right I think it is the loss of the habit of using the frozen section technique in arterial work that allowed this feature to be so long overlooked In a frozen section a mass of fibrin looks entirely different from what it does in a paraffin section

Dr Ambrus I would like to support Dr Duguid We did similar experiments in dogs with an entirely different purpose We have produced I[131] labeled fibrin clots to test fibrinolytic enzymes on these clots We found the clots would not dissolve after 4 days or so of treatment after they were produced When we treated them earlier we had good results We then produced a series of clots in blood vessels in dogs and cut them out at various time intervals for histological study After 3 or 4 days these clots became endothelialized This explains why fibrolytic enzymes were unable to dissolve older clots This is due to the lack of contact between the circulating enzyme and the fibrin fibers We followed each clot for a longer period of time and found exactly the same type of pictures which Dr Duguid showed in man

We have also taken I[131] labeled fibrin particles and injected them intravenously these wound up in the pulmonary circulation

Do you not think that similar processes in the vasa vasorum rather than in the main channels may be significant factors in intimal thickening and in what the pathologist reports as atherosclerosis?

Dr Duguid Yes but I think the actual intimal thickening is by the same process of surface deposit of fibrin

Chairman Lace How would you pass from the vasa vasorum and the deposition of fibrin to intimal thickening?

Dr Duguid I think almost anything happening in the region of an arterial wall seems to be conducive to the deposit of fibrin on the surface Although you may not be able to trace a connection between the vasa vasorum and the intimal surface in the aorta for example whenever you find a lesion in the vasa vasorum you will usually find a layer of fibrin on the surface

Dr Ambrus In experimental clots in dogs we have found that after a few days there is an invasion of fibroblasts and there is evidence these fibroblasts now deposit additional fibers which we did not feel qualified to classify If we follow these organized and endothelialized clots for a longer time some of the newer fibers appear like collagen they look different from the fibers which show up in the radioautogram as labeled fibrin fibers which we had injected originally I wonder whether you made any observations on cellular activity and subsequent fiber deposition in these arteries I beg your pardon if my questions are a bit naive but I am not a histologist

Dr Duguid That is all right We do not know how fibrin is converted into collagen whether it is an actual conversion of the fibers or a substitution

Chairman Lace We expect Dr Schmitt to answer that for us later

Dr Meyer Have you done experiments in which you replace fibrin or fibrinogen with another fibrous protein for example with collagen or elastin? To me the problem is this proliferation of fibroblasts occurs outside the blood vessels in many areas of connective tissue on the basis of either mechanical or specific chemical stimulation Why does this happen?

Dr Duguid I do not think one could begin to discuss the number of agencies

that excite fibroblastic proliferation The thrombus is only one The presence of lipids is another It is a reaction to the presence of any abnormal substance For example if a thread is run through an artery connective tissue proliferation occurs at the point where the thread runs through

CHAIRMAN PAGE Do you think this is really a major cause or mechanism for human atherosclerosis? I recognize this is a generality but in this country we are looking for a reorientation for new guide lines and if the older notion of infiltration with lipids and degeneration as the result of breakdown of lipids with stimulation of fibroblasts is not the correct one then we will have to start all over again

DR DUGUID I think surface thrombosis is of major importance in atherosclerosis but you mentioned experimental atherosclerosis

CHAIRMAN PAGE By that I mean the type resulting from the feeding of large amounts of lipids

DR DUGUID Dr Rannie and I (I Rannie and J B Duguid *J Pathol Bacteriol* 66 395 1953) published a paper in which we thought we produced fairly good evidence that in cholesterol sclerosis the process is essentially the same as in mural thrombosis We agreed with Leary that macrophages circulate in the blood and are deposited in heaps on the vessel wall and we demonstrated that the endothelium grows over the top of them so that they are incorporated in the same way as are mural thrombi

CHAIRMAN PAGE You will remember that Gofman published a paper in which he found no evidence from radioactive studies of the macrophages doing that You do not agree therefore with Gofman in that point of view?

DR DUGUID I am sorry I did not bring the preparations here to demonstrate my point

CHAIRMAN PAGE In other words you think it is perhaps a chemotactic accumulation of macrophages They stick onto the wall penetrate and then are covered over with endothelium?

DR DUGUID Yes but I do not think the macrophages actively penetrate the wall I think they are passively enveloped in it

DR WEXLER Dr Texon in New York City employs Bernoulli s principle to explain the mechanism of atherosclerosis A simple analogy would be if you stand in a shower and turn on the water the shower curtain will probably suck inward and flap against you According to Bernoulli s principle there is a main laminar flow which exerts suction You remember from your school days when you took a piece of bubble gum and stretched it out and exerted negative pressure you would pull the bubble inwardly Just so the intima is pulled out Dr Texon demonstrated that if the aorta is removed at the bifurcation of the iliac arteries the lesion would be on both sides of these vessels

Dr Texon also proposed that there should be an eccentricity to the lesions in the coronary vessels according to the trajectory of flow and thus the lesion should be on one side only

Recently my colleagues and I have produced an interesting variety of experimental arteriosclerosis in rats Without changing their diet these rats were made arteriosclerotic by making them hyperadrenocorticoid We have found three types of lesion in the coronary arteries one is intimal proliferation which has a few discrete foam cells the second is occlusion of the lumen with no foam cells the third (in agreement with Dr Duguid s hypothesis) is distention of the coronary vessels rather than occlusion The coronary vessels are greatly distended and in the media there is elastosis and light intimal calcification Therefore there is

similarity between some of these experimental lesions and those described by Dr Duguid

CHAIRMAN PAGE You would expect fibrosis to occur as the result of Bernoulli s principle acting on the wall In other words you get a stimulus for hyperplasia which would be the suction

DR WEXLER Yes physical stress

DR DUGUID I do not know about the suction Rindfleisch wrote on this subject before the beginning of the century discussing the mechanical effects and mapping out theoretically the points where atherosclerosis should hit the aorta but it was more the thrust of the blood flow that he suggested as the determining factor

CHAIRMAN PAGE Virgil Moon wrote a paper on the mathematics of turbulence I think and production of atherosclerosis

DR ALLEN Dr Duguid the thing that intrigues the clinician is that none of these considerations fits in well with clinical observation We have this problem of pulmonary arterial circulation which you may want to dispose of by saying the pressure is lower in the pulmonary circulation but as a pathologist you know that atheromatosis almost never causes occlusion of the radial artery and seldom a segmental occlusion of the subclavian artery In an extensive clinical experience I have never seen atheromatous occlusion of the radial artery which produced significant ischemia How about this? Also atheromatous occlusion of the major renal arteries and of the visceral arteries with sufficient atheromatosis to cause interference with the artery s function of transporting blood is very rare

DR DUGUID One reason for atheroma being unusual in the pulmonary artery is not so much because the blood pressure is lower as because the pulse pressure is lower I do not think mechanical aspects have much to do with the question of connective tissues but it is recognized that the arteries go through a fairly extensive movement between diastole and systole especially in hypertensives Atheroma is most prevalent in places where the greatest excursion of expansion and recoil take place Why thromboses should be more common in the long arteries of the leg than in those of the arm I cannot say The rate of blood flow may be a factor

DR ALLEN Since atheromatous occlusion of the arteries of the lower extremities is extremely common and of the radial artery is very rare I do not think we can group the long arteries of the legs and arms together

DR DUGUID Some atheromatous thickening of the radial artery occurs but never occlusion

DR ANGEVINE Dr Duguid how long does it take for these young thrombi you showed us to form In autopsy material there is always a danger that you see something that has happened during the last 2 or 3 days of life Material from accident cases etc would be more valuable perhaps Have you studied material from sudden deaths?

DR DUGUID Sir Howard Florey and his school have studied the speed of endothelialization and apparently it is rather slow Atheroma or any of the arterial lesions we are dealing with may be 20 or 30 years old You have to examine a lot of vessels pretty exhaustively before you can expect to find these lesions in the process of formation I admit they are difficult to find but nobody who set out to find them has reported failure Regarding the sudden death material I have examined a great many I manage to get nearly all the sudden deaths in Newcastle

DR RATNOFF Dr Duguid I wonder if this discussion does not beg a fundamental question Blood as it circulates in our vascular tree has the remarkable propensity for remaining unclotted or else we would all be very quickly solidified

This suggests to me that endothelial change must precede a fibrin clot Blood hitting the vessel wall would not make fibrin form unless there were something wrong with the endothelial surface What you see as a lesion may in fact be a secondary phenomenon

Has anyone done a sort of Coons experiment? One can imagine making perhaps in a nonmammalian species an antibody against fibrin This could then be applied to the vascular lesions which could then be counterstained with a fluorescent anti body which would indicate whether fibrin was present One would not have to worry about those electron microscopers who are only looking at little shadows

Dr Duguid Yes I admit your first implication but I haven t the slightest idea of what the primary change may be in the endothelium

Dr Shainoff Drs Vasquez and Dixon have prepared antifibrin labeled it and looked at the lesions of the generalized Schwartzman reaction Fibrinogen appeared to be a source of the lesion

Chairman Page Would you care to say anything about the problem of the relationship of coronary atherosclerosis and thrombosis? I think we would all agree that there is a common but not obligate relationship between the two This is of importance because in this country at least we tend in all of our epidemiological studies to equate myocardial infarction with coronary atherosclerosis

Dr Duguid In Jamaica some time ago I found that whereas atheroma of the aorta is extremely common in Negroes they never die of coronary thrombosis They have mural thrombi in their coronary arteries just as people have here or in Great Britain but they never have large thrombi The really crucial question is why thrombi sometimes grow too big

Dr Boucek Regarding Dr Duguid s hypothesis that degenerative changes in vessels should result in dilatation of the vessel arteries should not be considered to be similar throughout the body The Harknesses recently demonstrated differences in the amount of fibrous proteins of the dog arteries i e the aorta in the thoracic section was different from the abdominal aorta in its amount of elastin and collagen The thoracic aorta differed from the coronary or peripheral vessels chiefly in the presence of a higher elastin to collagen ratio in the peripheral vessel than in the thoracic aorta

Aging changes of the thoracic aorta usually result in an ectasia whereas in the peripheral vessel there is compromise of the lumen resulting from intimal thickening As Roberts and Moses have demonstrated early atherosclerotic changes occur to the greatest extent in the abdominal aorta in both sexes and in the coronary arteries of the male so that these atherosclerotic lesions may be related to the amount and the physical state of the fibrous protein of the vessel

Dr Duguid You must recognize the possibility that what you are calling fibrous proteins may not be part of the original vessel but may be derived from surface deposition

Dr Boucek No the work referred to was carried out on young dogs and biochemical quantitation of collagen and elastin was done

Chairman Page You are referring more to the physical state of the elastic tissues rather than the pathological term?

Dr Boucek Yes

Dr Adlersberg Dr Page what about other mechanisms resulting in changes of the arterial wall and probably relating to the process of atherogenesis? For instance Reinhart *et al* observed in pyridoxin deficient monkeys a marked thickening of the intima and eventually deposition of lipid I am also thinking of the ob

servations by Albertini in Switzerland and of Dr Zak and myself concerning areas of acute arteritis in the arterial wall with a superimposed thrombus as a possible mechanism of atherosclerosis especially in younger individuals In other words there are various pathological mechanisms which may result in thickening of the arterial wall and perhaps secondary infiltration with lipids The question arises is there any real relationship between the common variety of atherosclerosis as we see it daily in our patients and as we produce it experimentally in our animals by feeding cholesterol and some of these observations?

DR AMBRUS Dr Ratnoff brought up the question of vessel wall injury Dr Astrup will present his views on that later that there is a dynamic balance between continuous fibrin formation and fibrinolysis If that is acceptable it is not necessary to assume vessel wall injury It has been recognized for a long time that at least two factors are involved in the common type of atherosclerosis a high β lipoprotein level and a high blood pressure Either alone is sufficient or a combination of the two In this conference I think we are introducing other factors plus those Dr Adlersberg mentioned In any combination these factors can work together to produce atherosclerosis Yet there are many strange connections between these various factors For example it was shown by a number of workers that hyperlipemia may inhibit the inherent fibrinolytic activity of blood which would then tip the dynamic balance in the direction of fiber deposition

CHAIRMAN PAGE I think everyone would agree that the mechanism of atherosclerosis is multifaceted Unfortunately each mechanism must be taken out of the whole to be studied This does not mean that they do not act in concert to produce the completed picture of atherosclerotic cardiovascular disease

DR WEXLER Dr Duguid have you correlated the age of the individual and morphology of the lesion? You spoke about the Jamaica people and their coronary vessels How about the age correlation in England or Newcastle?

DR DUGUID So far as I know there is no correlation between the age incidence of atherosclerosis of the aorta and coronary artery atherosclerosis We have not studied the question systematically but Morris has of course in coronary atherosclerosis and I think his data agree absolutely with the figures in America I believe it increases progressively as one gets older

DR WEXLER What do you see in the very young? Do you see lesions of this sort at all?

DR DUGUID Yes

DR MEYER I have no quarrel with Dr Duguid's theory as a possible factor in atherosclerosis but it seems to me this theory is based on an abnormality in the blood coagulation of these people Is there any correlation between atherosclerosis as you observed it and a history which points up to a failure or a speed up of blood coagulation?

DR DUGUID No I think such a correlation would be difficult in a chronic disease such as atherosclerosis When I first started to look for arterial thrombosis I chose cases in which there was some history suggestive of thrombosis for example men who had died from coronary thrombosis and I usually found such men had multiple thrombi elsewhere in their circulation besides the coronary arteries That however merely indicated a tendency to thrombosis at or immediately before death and had no bearing on the state of affairs 20 or 30 years previously when their atherosclerotic lesions may have developed The tendency to thrombosis may be a transient and variable affair and we would have to trace its variations throughout life to make an effective correlation with atherosclerosis

DR RATNOFF Regarding Dr Meyer's question unless I am confused there is no evidence that any clinical state presently defined has intrinsically accelerated blood coagulation associated with formation of thromboses The thrombotic disease cannot to my knowledge be satisfactorily correlated with *in vitro* evidence of what you might call increased coagulability At least ten papers a month claim such a phenomenon in one disease or another or one physiological state or another One week it is ice cream cones and all that lipid and next week something else Always when such experiments are repeated no increased coagulability is demonstrable *in vitro* Any hypothesis based on available data rather than on wishful thinking has to assume that something extrinsic to the circulating blood initiates the thrombotic process Perhaps in the future there will be some way of showing an intrinsic change in the blood itself I know of no evidence that there is ever hypercoagulability" in the blood itself

DR ALLEN Dr Duguid can you extend the story of pulmonary emboli to show that in these lesions produced by the method described there is in fact deposition of lipid material and calcification?

DR DUGUID Certainly a small amount of lipid but not very much calcification

DR WHITE Is one restricting the situation a bit particularly experimentally by limiting thrombus definition to deposition of fibrin? One may have thrombus deposition which may be unrelated to the blood clotting mechanism i e to thrombin but may be literally a deposition of large amounts of insoluble lipoprotein or lipid at the site of vascular injury This relates perhaps to the generalization that Dr Meyer referred to namely whether in circumstances of fibroblastic proliferation subsequent to an injury one could not have deposition of material which would interfere with the elasticity of the vessel unrelated to the deposition of fibrin but nevertheless a thrombus

DR DUGUID I am firmly of the opinion that thrombosis is different from and possibly quite independent of coagulation You will time and again see in persons who have died from coronary thrombosis thrombi confined to short lengths of the coronary arteries and beyond them columns of blood which although stagnant are perfectly fluid That takes a lot of explaining on the coagulation theory

I don t think the hyaline material which you see in the arterioles is necessarily fibrin it looks more like a protein coagulum of some kind

DR JACKSON Talking about electron microscopy I was a bit puzzled about your difficulty in identifying fibrin—Dr Schmitt probably knows more about it—but I think it is a slur on the electron microscopists of Britain to say they couldn t identify it because a number of people do

CHAIRMAN PAGE You may recall that Levene in England thought he had produced convincing evidence from electron microscopy that although the fibrous tissue forming atherosclerotic plaques appears with ordinary stains under the light microscope to be collagen it actually was fibrin The fibrin was the residue to a mural thrombus However other equally British microscopists did not agree The question is then whether an expert such as Dr Schmitt can resolve the problem for us

DR SCHMITT If fibrin is appropriately prepared it shows in the electron microscope a characteristic band pattern with an axial repeat of 235 A In tissue especially in preparations which have not been especially treated fibrin may appear as tactoidal or irregular fibrillar structures It would be difficult to prove that such fibrils are indeed composed of fibrin The preparation would have to be very dirty indeed if the collagen fibrils cannot be identified by their band pattern

I must also confess that as a biologist I tend to consider the blood vessel as a complex system in its own right The deposition of a substance such as a steroid or of fibrin is but an event in a very complex process that eventually leads to the clinical phenomenon We should be sensitive of the many factors involved in the system as a whole rather than focusing attention exclusively on a few substances that seem to be intimately involved The situation reminds one of the tendency after Spemann s classic work on developmental biology to seek to identify the magic substances (evocators organizers) thought to be responsible for the regular unfolding of the organism This biological myopia was soon cured by the discovery that many substances could act as evocators or organizers That which was specific was the system as a whole To understand this we must learn a good deal more about the molecular biology of the developing embryo

I have a suspicion that the processes involved in the occlusion of a blood vessel may similarly depend on the reacting cellular material and tissue and that these processes may be triggered off by many types of substances themselves relatively nonspecific

DR HARTROFT Our work is a direct outgrowth of Dr Duguid s Two or three years ago after Dr Duguid had published his findings Dr W A Thomas and Dr R M O Neal in our laboratory at Washington repeated and confirmed his work with rabbits They were struck with the fact as Dr Duguid mentioned that little fat was present in the lesions that resulted from organization of injected thrombi To accomplish the histological objective of incorporating fat into these lesions if possible in subsequent experiments the animals injected with thrombi were stomach fed large amounts of butter in addition to being offered the stock diet which contained but little fat only about 1%

When the lesions in these rabbits were examined fat had become incorporated into the organized thrombi—which was the objective of the experiment But the unexpected finding was the much higher frequency and extent of lesions As Dr Duguid mentioned far more thrombi must be injected than the number that persist as organized fibrous intimal thickenings But when meals of butter were given more and larger lesions resulted This experiment was repeated several times using isocaloric syrup as a control and different types of fat in other groups of animals Corn oil did not give this enhancing effect in the production of lesions whereas butter and oleomargarine did repeatedly We became convinced that the saturated fats that we had fed were interfering with lysis of injected clots so that those entrapped in the vessels of the lung survived in greater numbers and produced larger lesions of intimal fibrosis

With Dr Sol Sherry s help several other experiments have been done since both *in vivo* and *in vitro* to demonstrate this effect I will not go into detail because I am sure Dr Astrup will discuss this.

Connective Tissue Reactions in the Development of Arteriosclerosis*

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Studies by a number of investigators have dealt with the role of the connective tissue elements in the development of arteriosclerosis (3, 4, 7, 9, 10). The present report is concerned with the reactive alterations in endothelium, fibroblasts, intercellular fibrillae and ground substance of the coronary arteries in arteriosclerosis and with certain systemic factors which may influence these elements of the arterial wall.

I. NORMAL ARTERIES

Normal or "ideal" arteries are characterized by a single layer of endothelial cells which lie on an intact internal elastic membrane. The internal elastica is a conspicuous layer of homogeneous material as observed by both light and electron microscopy. In studies with electron microscopy it may be observed that the elastic tissue is of moderate density and presents a homogeneous appearance; the elastic tissue of the media forms a rich anastomosing network lying between the smooth muscle cells and forming connections to the internal elastic membrane as well as to the elastic tissue of the adventitia. Small amounts of mucopolysaccharide are present beneath the endothelium and in the media. This material is closely associated with elastic tissue and collagen and surrounds these fibers. Fibrocytes or fibroblasts are present in small numbers in the intima and media; the occurrence of mucopolysaccharide and collagen in these layers is a manifestation of the function of these cells. The adventitia contains mucopolysaccharide, fibrocytes, delicate elastic fibers, and abundant collagen.

II. SCLEROTIC ARTERIES

The histologic processes observed in the development of arteriosclerotic lesions in muscular arteries are essentially identical with the reparative processes of connective tissues following injury. We have

*These studies were supported by Research Grants H 1542 and C 2155 of the U. S. Public Health Service.

I must also confess that as a biologist I tend to consider the blood vessel as a complex system in its own right The deposition of a substance such as a steroid or of fibrin is but an event in a very complex process that eventually leads to the clinical phenomenon We should be sensitive of the many factors involved in the system as a whole rather than focusing attention exclusively on a few substances that seem to be intimately involved The situation reminds one of the tendency after Spemann s classic work on developmental biology to seek to identify the magic substances (evocators organizers) thought to be responsible for the regular unfolding of the organism This biological myopia was soon cured by the discovery that many substances could act as evocators or organizers That which was specific was the system as a whole To understand this we must learn a good deal more about the molecular biology of the developing embryo

I have a suspicion that the processes involved in the occlusion of a blood vessel may similarly depend on the reacting cellular material and tissue and that these processes may be triggered off by many types of substances themselves relatively nonspecific

DR HARTROFT Our work is a direct outgrowth of Dr Duguid s Two or three years ago after Dr Duguid had published his findings Dr W A Thomas and Dr R M O Neal in our laboratory at Washington repeated and confirmed his work with rabbits They were struck with the fact as Dr Duguid mentioned that little fat was present in the lesions that resulted from organization of injected thrombi To accomplish the histological objective of incorporating fat into these lesions if possible in subsequent experiments the animals injected with thrombi were stomach fed large amounts of butter in addition to being offered the stock diet which contained but little fat only about 1%

When the lesions in these rabbits were examined fat had become incorporated into the organized thrombi—which was the objective of the experiment But the unexpected finding was the much higher frequency and extent of lesions As Dr Duguid mentioned far more thrombi must be injected than the number that persist as organized fibrous intimal thickenings But when meals of butter were given more and larger lesions resulted This experiment was repeated several times using isocaloric syrup as a control and different types of fat in other groups of animals Corn oil did not give this enhancing effect in the production of lesions whereas butter and oleomargarine did repeatedly We became convinced that the saturated fats that we had fed were interfering with lysis of injected clots so that those entrapped in the vessels of the lung survived in greater numbers and produced larger lesions of intimal fibrosis

With Dr Sol Sherry s help several other experiments have been done since both *in vivo* and *in vitro* to demonstrate this effect I will not go into detail because I am sure Dr Astrup will

ture and degeneration of the internal elastic membrane may be the result of interference with the metabolic processes concerned with maintenance of elastic tissue

2 *Phase II*

In the second phase of the reaction of the arterial connective tissue the proliferative lesion undergoes maturation The internal elastic membrane is still present so that there appears to be a splitting or reduplication of this layer In addition many delicate elastic fibrillae form in the subendothelial tissue The origin of elastic tissue and its relationship to mucopolysaccharide is not clear although the elastic tissue is apparently formed in a matrix of mucopolysaccharide In most areas the elastic tissue is surrounded by a delicate layer of sulfated mucopolysaccharide which is resistant to the enzymatic action of hyaluronidase

Collagen fibers develop in the matrix of the mucoid ground substance The fibroblasts become converted into fibrocytes and there is a decrease in the amount of mucopolysaccharide Endothelial lined channels are not present except in those instances where a large far advanced lesion has developed as a result of multiple episodes of the reactions described above

3 *Phase III*

The third phase of the reaction is essentially degenerative It is characterized by the lack of active response of the connective tissue elements The fibrous connective tissue laid down during the preceding phases becomes acellular the collagenous and elastic tissue undergo degeneration and hyaline material replaces the previously organized tissue Lipid and frequently calcium appear in the areas of degeneration calcium is first observed in the region of the internal elastic membrane Endothelial lined channels make their appearance adjacent to the degenerated areas

The above processes may occur repeatedly over a period of years frequently in the same areas of the involved artery so that in many instances one may observe more recent reactions superimposed on areas that have become mature or undergone degeneration In this manner the older portions of a sclerotic plaque come to lie adjacent to the media whereas the more recently formed areas lie directly beneath the endothelium

The causes of degeneration of connective tissue remain unknown The nature of and the mechanisms involved in hyalinization are still obscure likewise the significance of the lipid in atheromatous plaques is unknown In our studies the lipid concentration is maximal in the

studied these reactions in detail in the coronary arteries The sequence in which the various processes occur is more or less orderly However in most instances the sclerotic lesions present a complex picture of multiple processes which overlap each other and coexist in the same area This is further complicated by the fact that the early phase of the connective tissue reaction is often superimposed on lesions which have developed previously and hence are composed of mature or degenerating scar tissue

The reactions of the arterial connective tissue may be divided chronologically into three phases these are namely (*1*) initial reaction to injury (*2*) maturation of the fibrous scar and (*3*) degeneration or senescence of the fibrous connective tissue The sequence of these phases is therefore similar to the healing processes in other tissues of the body In young individuals the degenerative phase is not observed except in rare instances

1 *Phase I*

The initial reaction of the arterial wall is characterized by several more or less simultaneous alterations Rupture fragmentation and fraying of the internal elastic membrane is consistently present this is the most obvious evidence of damage to the vessel Associated with this *there is increased production of mucopolysaccharide* by the subendothelial fibroblasts adjacent to the site of rupture of the internal elastic membrane Increased amounts of mucoid ground substance appear also in the media beneath the site of the ruptured segment of elastic tissue The mucoid ground substance contains large amounts of acid mucopolysaccharide Most of the ground substance may be removed by treatment with hyaluronidase however small amounts of residual sulfated mucopolysaccharide may be found in close relationship to elastic tissue It has not been possible to demonstrate alterations in this sulfated mucopolysaccharide Proliferation of the subendothelial fibroblasts is an important feature in the early phase of development of sclerosis Endothelial proliferation may also occur but is not a prominent feature

The close association between rupture and degeneration of the internal elastic membrane the formation of acid mucopolysaccharide and the proliferation of fibroblasts suggests that these processes are causally related Rupture of the internal elastic membrane may represent the initial tissue response to an abnormal hemodynamic situation If this is true then the production of mucopolysaccharide and proliferation of fibroblasts constitute the mechanism for regeneration of the internal elastic membrane On the other hand if the increase in mucopolysaccharide and fibroblastic proliferation occurs as primary processes rup

In experimental animals the proliferation of the fibroblasts and production of hyaluronic acid are depressed by adrenal cortical steroids The inhibitory effect of cortisone and hydrocortisone on the healing of wounds has been adequately demonstrated (1 13) Inhibition of growth and function of fibroblasts by these substances has also been demonstrated in tissue culture (2 6 8) Thus it seems likely that the proliferative response of arterial connective tissue may be influenced by adrenal cortical function

Recently we have reported on the effect of pituitary growth hormone on the rate of growth of fibroblasts in tissue culture (12) In these studies it was observed that fibroblasts grown in media containing somatotropin increased in number and synthesized protein more rapidly than controls Further studies have been conducted on the effect of growth hormone on hyaluronic acid of the dermis in experimental animals These studies have demonstrated that there is a direct relationship between the concentration of growth hormone and the hyaluronic acid content of the dermis It was also observed that the mucoprotein levels of the blood responded similarly to the administration of growth hormone Therefore it seems likely that pituitary growth hormone exerts a significant effect on proliferation of fibroblasts and the production of hyaluronic acid in arteries

IV Summary

The development of arteriosclerosis is essentially identical with reparative processes of connective tissues The reactions may be divided into three phases namely initial reaction to injury maturation of the scar and senescence of the fibrous connective tissue The initial reaction consists of rupture and fragmentation of the internal elastic membrane deposition of increased amounts of acid mucopolysaccharide and proliferation of subendothelial fibroblasts and endothelial cells In the second phase the internal elastica regenerates collagen fibers are laid down in the mucoid ground substance and the fibroblasts become converted to fibrocytes In the third phase the connective tissue elements undergo degeneration lipid and calcium appear and capillaries develop adjacently to the areas of degeneration The above processes may occur many times in the same area

The development of arteriosclerosis may be regarded as general biologic phenomena of reaction of arterial connective tissue to various stimuli However it seems likely that systemic factors e g nutritional and endocrine influence the degree of response of the vascular wall and the rate of development of sclerotic lesions

older portions of arteriosclerotic plaques adjacent to the former internal elastic membrane This suggests that the formation or deposition of lipid in the fibrous connective tissues of plaques is a degenerative or senescent process occurring primarily in lesions of long standing However it is also well established that intimal deposits of lipid may also be derived from the blood stream

III Some Systemic Factors Modifying the Development of Arteriosclerosis

The onset of arteriosclerotic changes early in life (5 11) and the remarkably high incidence of these lesions indicate that these connective tissue reactions are general biologic phenomena It seems reasonable to regard these reactions as nonspecific responses to various types of stimuli However the great variation in severity from one individual to another even in the same age group suggests that there are systemic factors which modify the degree of response of the vascular wall and the rate of development of these lesions

In view of the fact that reactions of connective tissue are an integral part of the development of arteriosclerosis it seems desirable to consider the roles of some of the factors known to affect the growth and function of connective tissues Specific nutritional deficiencies have been shown to have a profound effect on connective tissues It has been known for many years that ascorbic acid deficiency results in defective formation of connective tissue ground substance It has been observed that chronic deficiency of pyridoxin in the rhesus monkey results in the development of generalized arteriosclerosis similar to that observed in the human (14 15) These lesions are characterized by deposition of mucoid ground substance degeneration and regeneration of elastic tissue proliferation of subendothelial fibroblasts and the formation of collagen The precise mechanisms involved in the development of arteriosclerosis in pyridoxin deficiency have not been elucidated However it has been established that pyridoxin deficiency interferes with *transamination and transsulfuration* *Inasmuch as both of these processes* are intimately concerned with protein metabolism defective formation and maintenance of elastic tissue and collagen may be factors of major importance

The modification of the response of connective tissues to injury by endocrine factors is well known however their role in arteriosclerosis still requires clarification Among the hormones which have been demonstrated to have a significant influence on fibroblasts and ground substance are the adrenal cortical steroids and pituitary growth hormone

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Discussion

Dr Angevine In all the injuries did you see anything to suggest thrombosi in the early developing lesions?

Dr Moon Not in the younger age group Thrombosis was observed only in individuals 30 or 40 years or older with severe or far advanced arteriosclerosis In some cases it would be possible to find organizing thrombi

Dr Angevine In these very young lesions at what time did the elastic fiber begin to appear?

Dr Moon They begin to appear at about the same time as the collagen fiber do I do not know how long the lesion had been present before these fibers developed

Dr Gitlin I might point out something that perhaps pathologists do not seem to realize that there is another way to view collagen The fluorescent microscope is extremely valuable in looking at the elastic membranes and the formation of collagen Have you had an opportunity to study these lesions under the fluorescent microscope?

Dr Moon No we have not In the limited number of vessels we have studied by electron microscopy collagen can be readily identified and elastic tissue appears as a rather homogeneous moderately dense material

Dr Meyer Dr Gitlin does your observation about the fluorescent microscope apply to very young elastic tissue or only to mature or older elastic tissue?

Dr Gitlin It applies to both

Dr Meyer Even in the very young?

Dr Gitlin It has a very blue fluorescence It is the most striking thing in any tissue It is the quickest way to detect elastic tissue or collagen

Dr Meyer Have you distinguished between collagen and elastic tissue?

Dr Gitlin Only by quality The elastic tissue has a much brighter fluores

ence than does the collagen in the very young I do not ordinarily study older people

Dr Hartroft Regarding the distribution of lipid that Dr Moon showed in well-developed lesions I certainly agree the lipid is deposited most frequently near the internal elastic lamina at the base of the lesion furthest from the vessel lumen In a variety of aortic lesions obtained from young and old people that we have studied including both small lesions and larger ones we have been impressed with the variability of the distribution of the lipid

We are also struck by the scarcity of foam cells in lesions of the aorta I think the foam cell is more famous than it should be as a constituent of plaques in the aorta although this impression may not hold for the coronary arteries In many aortic lesions a stratification of deposits is often apparent in sections stained for lipids with a zone containing fat alternating with the next which may be relatively free of lipids This appearance irresistibly suggests that such zoning may have resulted from a series of separate events responsible for the formation of these lesions lipid being sometimes deposited and sometimes not

If one does subscribe to the thrombogenic theory of atheroma one can imagine that a series of superimposed mural thromboses might have occurred whether or not they were the initiating events The amount of fat deposited each time might merely reflect the degree of lipemia present at the time thrombosis occurred thereby producing this layered effect because of variation in the amount of lipid deposited in each resulting zone

Dr Moon No I don t think so

Dr Glimcher As to Dr Meyer s question about hyaluronic acid and chondroitin sulfate there is an article in the current *Biochimica et Biophysica Acta* on the separation by chromatography and electrophoresis of acid mucopolysaccharides from arteries (aorta)

Dr Meyer That would not be of much use for histological identification

Dr Glimcher No but it gives you an idea in what proportion the various mucopolysaccharides are present—whether or not one particular fraction was there at all!

Dr Meyer We have done that by alcohol fractionation and I believe this would not be changed by chromatography that the major proportion of the total acid mucopolysaccharides can be extracted after proteolytic digestion of the whole tissue as chondroitin sulfate and I believe it is the type we call A that is the polysaccharide which is the main component of cartilage and bone

Dr Moon Dr Meyer in the formation of collagen what is the mucopolysaccharide?

Dr Meyer I do not know that it has been studied Judging from what little work has been done on tissue culture both in our laboratory and in others the major component of tissue culture fibroblasts and of embryonal growths is hyaluronic acid and there is a small amount in all fibroblasts that we have investigated There is a small amount of sulfated polysaccharide which appears to be C This combination of hyaluronic acid and chondroitin sulfate C seems to be characteristic of all rapidly growing young fibroblasts In aging this changes

Dr Moon In the interpretation of our finding I was using the evidence that fibroblasts in tissue culture did produce hyaluronic acid

Dr Meyer I think it has to be proved that this is hyaluronic acid There are other methods which you could investigate as to whether it is hyaluronic acid or not

DR JACKSON The process seems to vary with the situation—whether there is polysaccharide before collagen In tissue produced in the sponge long after collagen is being formed polysaccharide is almost impossible to find There is a very minute amount of hyaluronic acid and chondroitin sulfate but most of the hexosamine is probably serum protein A similar situation appears to exist in the carrageenin granuloma

In general I believe it is not necessary to have polysaccharide present to have collagen formed I see no causal relationship between the two phenomena

DR GITLIN But if these sections were digested with hyaluronidase where would it occur?

DR JACKSON I am not suggesting it occurs this way The polysaccharide does appear in some instances but collagen [does] forms in the absence of polysaccharide

DR MEYER I would like to see the evidence for this

DR JACKSON In the sponge and in the carrageenin granuloma one can isolate all the collagen fractions 8 or 10 days after the thing has been formed Neither at that stage nor before can you find polysaccharide

DR MEYER The quantity of polysaccharide at best would be perhaps 5% of the total dry weight of tissue

DR JACKSON Yes that is true

DR MEYER What would be your recovery of polysaccharide if you dealt with a few hundred milligrams of sponge tissue?

DR JACKSON In the sponge we can produce as much as 5 gm of connective tissue I can demonstrate in terms of the proportion of hexosamine that the vast majority of hexosamine can be definitely identified with the serum mucoprotein by isolation of the serum mucoprotein and analysis of total hexosamine and hexosamine isolated as serum glycoprotein Simultaneously acid polysaccharides will account for less than 5% of the hexosamine After papain digestion which in other situations removes most of the hexosamine there is still about 20% left If all the hexosamine is added this would be about 95% of the total hexosamine so I cannot see that I am losing much

In the granuloma with up to 500 gm of connective tissue 10 mg of hyaluronic acid can be isolated and the same amount of chondroitin sulfate from a hundred grams of tissue whereas a lot of serum mucoprotein is obtained in the same situation

DR MEYER I believe this is correct that the main part of the hexosamine is in serum or comes from serum proteins but I would still like to see the evidence showing that collagen develops in the absence of mucopolysaccharide

DR JACKSON Dr Boucek you measure galactosamine do you not?

DR BOUCEK Dr Noble in our laboratories has not detected galactosamine until after the tenth day following implantation of the sponge in male rats The hexosamines in the early connective tissue containing collagen is glucosamine presumably derived from glycoprotein and hyaluronic acid

DR JACKSON I would like to protest the use of ground substance and mucopolysaccharide synonymously A vast proportion of the ground substance is not of mucopolysaccharide origin

DR SHERRY Dr Moon commented on the influence of growth hormone and of steroid hormone on the development of the atherosclerotic lesion Would somebody comment on the influence of insulin on the metabolism of polysaccharides?

DR WHITE Yes Dr Sara Schiller and Dr Albert Dorfman have published a

paper on the biosynthesis of mucopolysaccharides in the alloxanized diabetic rat as influenced by the administration of insulin (S Schiller and A J Dorfman *J Biol Chem* 227 625 1957) In the alloxanized diabetic rat there is an inhibition of mucopolysaccharide formation as evidenced by the diminished rate of incorporation of labeled glucose and labeled sulfate this rate is restored toward normal if the alloxanized diabetic rat is administered insulin The data are interpreted in terms of the susceptibility of the diabetic animal to infection as compared with the normal

Dr. Gitlin The trouble with these animals is that so many things happen in diabetes Even protein synthesis is affected There seems to be a general decrease in synthesis of a great many things in the alloxanized animal It is not a specific effect as far as I can see

Dr White It is not a question of a specific effect The point to be made is that the alloxanized diabetic rat has an inhibited capacity to form mucopolysaccharide regardless of what else is happening

The Macromolecular Basis of Collagen Structure*

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For many years collagen and the connective tissue generally were rather uninteresting to the physiologist biophysicist and biochemist As a kind of stuffing excelsior it was known to enclose and support the various tissues and in special forms to provide particular mechanical properties e g tendons for high tensile strength in bones as an organic matrix upon which to build the sturdy mineralized skeleton and in teeth a similar function The pathologist has long been interested in collagen not only as the basis of the so called "collagen diseases but also because of the central role of collagen and the connective tissue in the rheumatoid diseases in cardiovascular disease and indeed in the process of aging itself However it is doubtful that even such a formidable array of clinical implications would have caused basic scientists to become interested in the subject it is still rather complex for definitive attack by biophysicists and biochemists One reason for the difficulty lay in the fact that collagen occurs primarily in fibers that are insoluble

This situation has changed very radically in the last decade or two Collagen is in fact one of the best known of fibrous proteins today It is studied by crystallographers physical chemists and electron microscopists as a model of fibrous protein To some extent this is due to new developments in the interpretation of X ray diffraction patterns of collagenous tissue It turns out that the collagen class of proteins as Astbury (1) first categorized them is unique in possessing three covalent chains helically coiled upon themselves to form the macromolecule† of collagen (hereafter to be referred to as "tropocollagen" abbreviated TC) It is in this form or as a closely similar precursor thereof that collagen is synthesized in the fibroblast It is presumably

These studies were aided by a research grant from the National Institute of Allergy and Infectious Diseases National Institutes of Health U S Public Health Service

† The unit of native collagen structure is referred to as a macromolecule rather than a molecule because it appears to be composed of several covalent polypeptide chains bonded together by hydrogen bonds

in this form that the very thin highly elongate macromolecules find their way in physical solution in the saline milieu of the connective tissue ground substance It is in this form that the macromolecules polymerize end to end to form protofibrillar polymers which after association literally form the collagen fibrils seen in the electron microscope and the larger fibers seen in the light microscope It is therefore obvious that to understand the physiology and the pathology of the connective tissues one must first investigate and understand the properties of the tropocollagen macromolecule It is about this subject that the present paper is concerned

It so happens that a number of rather vital tissue constituents have similarly proven to be very thin highly elongated macromolecules (e g myosin paramyosin tropomyosin actin) To understand the forces that act between these macromolecules and the special interaction characteristics of such systems the basis of tissue function has therefore come to be an important goal in molecular biology

By virtue of their particular amino acid composition collagen fibrils manifest a certain highly characteristic banded structure as seen in the electron microscope By an analysis of this structure it has been possible to deduce not only the nature of the constituent tropocollagen macromolecules but also the factors involved in causing these macromolecules to aggregate in characteristic fashion The collagen system has therefore come to interest all those concerned with tissue functions that are essentially fibrous

In this paper we present certain of the more pertinent facts concerning collagen at the molecular (or macromolecular) level These facts may be of interest not only to those concerned with connective tissue problems but also to molecular biologists interested in other types of biological problems that involve specificity of interaction of elongate macromolecules

I The Biophysical and Biochemical Properties of Collagen

In contradistinction to most proteins collagen (or perhaps more correctly the collagen class) possesses very characteristic structural and chemical properties which permit its definitive identification For present purposes we shall sketch very briefly only those properties necessary for an understanding of the internal structure and the chemical properties of the collagen macromolecule For recent excellent surveys of these properties see Gustavson (16) Highberger (23) Tunbridge (42) and Stainsby (39) in "Recent Advances in Gelatin and Glue Research"

Collagen occurs in dense fibrous tissue of high tensile strength as in tendons or less tightly woven tissue fabrics as in skin or in more

sparse distribution as in loose connective tissue The fibrous protein occurs in various hierarchies of fiber size as shown in Fig 1 taken from the excellent review of Bear (4) These include the following *fibers* visible macroscopically or microscopically and having diameter of the order of micra *fibrils* with widths of the order of a few hundred

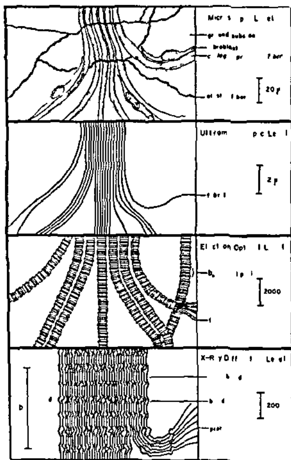


FIG 1 Hierarchies of structural elements of fibrous collagen in increasing magnification From Bear (4)

to several thousand Ångstrom units observable in the dark field microscope and resolvable in the electron microscope *protofibrils* which were originally defined as constituting "The unit columnar arrays which when associated laterally form the collagen fibril and the tropo

collagen *macromolecule* which constitute the monomeric units of the protofibrillar polymer.

For X-ray diffraction and for chemical analytical studies, gross macroscopic fibers or whole tissues are used. For electron microscopic investigation the fibrous category of interest is the fibril which manifests a detailed and characteristic band pattern which, as will be brought out below, results from a specific pattern of aggregation of the elongate native collagen macromolecules.

The collagen class of proteins is uniquely characterized by their amino acid composition, their X-ray diffraction pattern, and their banded appearance in the electron microscope. These characteristics may be very briefly described as follows.

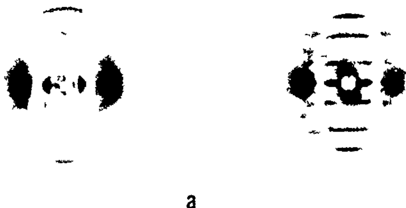


FIG. 2. Large angle X-ray diffraction patterns of collagen from rat tail tendon. After Randall (33a). a, unstretched. b, stretched 8%.

The collagenous proteins differ from other proteins in that they contain the amino acids hydroxyproline and hydroxylysine. In mammalian collagen about one third of the amino acid residues are glycine. Proline and hydroxyproline together make up almost another third, leaving approximately one third for other amino acid types. From a determination of the hydroxyproline and glycine content of a given preparation one can estimate the collagen content.

Perhaps the most distinctive characteristic of collagen is its large angle X-ray diffraction pattern which reflects the internal organization of the collagen macromolecule and is therefore characteristic of this

class of proteins Astbury (1) early called attention to the 2 86 A meridional reflection which he considered to represent the length of the amino acid residues along the fiber axis in the coiled polypeptide chain and to the equatorial reflections at 10–16 A (depending upon the degree of hydration) which he attributed to the lateral separation

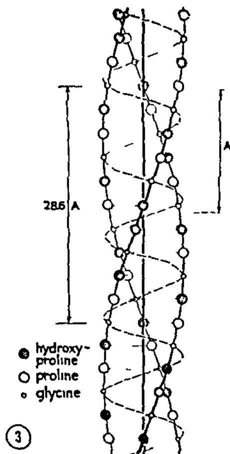


FIG 3 Three stranded helical structure of collagen macromolecules Courtesy of Dr A Rich

between main chains With more refined technique it has been possible to obtain far more reflections in the pattern and to achieve a higher degree of orientation by stretching fresh tendon From such patterns (see Fig. 2) several groups of workers agreed that the diffractions are best interpreted in terms of a macromolecule containing three chains

coiled in helical fashion about one another to form a coiled coil [see particularly the papers of Crick and Rich (7 8)] The proposed triple stranded structure is shown schematically in Fig 3

It has also been proposed that only two types of three stranded helical models of collagen structure need be considered based on the so called Structure I and Structure II derived from a consideration of polyglycine and compatible with the X ray infrared analytical and physicochemical data In these models the axial repeat occurs at 28 6 A In Collagen II thought to agree best with the diffraction data the OH groups of the hydroxyproline residues extend radially from the three chains making it possible to form hydrogen bonds with CO groups of adjacent three stranded macromolecules In the Collagen I structure the hydrogen bonds from hydroxyproline are internally directed bonding the three chains intramolecularly The hydroxyproline content appears to be determinative of the denaturation temperature which is a measure of the energy needed to disrupt the internal organization of the macromolecule This fact tends to support the Collagen I type of structure Rich has suggested that one type of structure might be convertible into the other and that this may result from application of stress to the fiber

Although there is fairly general agreement that the collagen macromolecule is a three stranded helix it is not certain that the macromolecule is thus constructed over its entire extent Gallop (personal communication) suggests that a substantial portion may have a different configuration

Treatment of soluble collagens with hydrogen bond breakers like urea or by heating causes denaturation with the liberation of the constituent chains to form parent gelatin From the original macromolecule having a weight of 360 000 there is formed according to Doty and Nishihara (1958) one chain with a weight of 120 000 and another of weight 240 000 These authors believe that an alkali labile ester bond links two chains of weight 120 000 to form the heavier chain obtained from denatured collagen This is to be compared with the corresponding values of Orekhovitch and Shpikiter (33)

In addition to the large angle X ray pattern arising from the internal presumably three stranded structure of the macromolecule collagen also manifests a well developed small angle X ray pattern (Fig 4) consisting of many (about 50) orders of a large axial repeat which Bear (3) showed to be 640 A in air dried fibers and nearer to 700 A in moist fibers Although all native collagen fibers from a wide variety of sources showed this axial repeat its significance in terms of molecular structure was not obvious The simplest early interpretation was that

it represents the molecular length of the collagen molecules an assumption that seemed to gain support from the fact that a similar axial repeat was observed in the band pattern in electron micrographs (36) Bear and Morgan (5) attempted to relate the positions of the intraperiod bands observed electron optically with the characteristic intensity of the various orders of the small angle X ray pattern As will

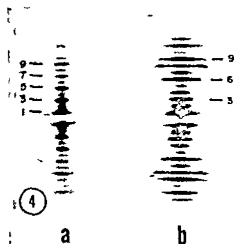


FIG 4 Small angle X ray diffraction patterns of kangaroo tail tendon collagen a moist preparation b after brief exposure to water and drying under tension Layer line indices are indicated After Bear *et al* (5a)

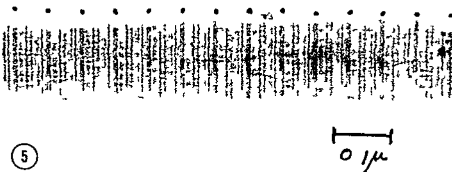


FIG 5 Electron micrograph of calfskin collagen reconstituted from solution stained with phosphotungstic acid Band pattern is of the native type Axial repeats marked Courtesy Dr A J Hodge

be shown below the collagen macromolecule very probably has a length of four times the 700 A period i e about 2800 A

The band pattern observed in high resolution electron micrographs of teased collagen fibrils stained with phosphotungstic acid (PTA) or other heteropoly acid is characteristic of collagen (see Fig 5) This axial pattern repeats at about 700 A and contains a number of bands and interbands of characteristic density and position It was suggested by Bear (4) that the bands represent regions of relative disorder due to the interaction of side chains of relatively large size while the inter

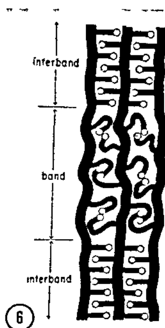


FIG 6 Interpretation of band and interband regions according to Bear (4) Vertical chains represent collagen macromolecules whose side chain interactions *produce bands and interbands*

bands represent regions of relative order due to the interaction of the smaller side chains which are found in considerable abundance in collagen (see Fig 6) Another interpretation of band structure depends upon the characteristic interaction of groups such as the guanidino groups of the arginine side chains with PTA as suggested by Kuhn *et al* (29) It will be noted from Figs 5 and 7 that the band pattern i e the intraperiod positions and the relative densities of the bands is a polarized asymmetric pattern The significance of this pattern was discovered only after it became possible to take the native fibrils apart

into their constituent macromolecules and to cause these to re aggregate in characteristic and new band patterns These results may now be briefly described

II Formation of Ordered Aggregation States of Collagen by Precipitation from Solution

A formidable difficulty in the characterization of the collagen molecules lay in the relative insolubility of collagen fibers However certain types of collagen such as in rat tail tendon and in the fish swim bladder are soluble in dilute acid From the classical early work of Zacha

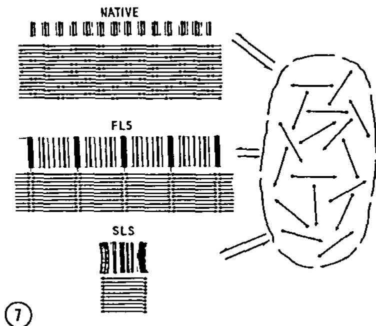


Fig 7 Diagrammatic illustration of patterns of aggregation of tropocollagen macromolecules in native FLS and SLS types Polarization of macromolecules indicated by arrow

riades Nageotte Faure Fremiet Wyckoff and Corey and others it is known that by appropriate adjustment of the pH and ionic strength of such acid solutions the collagen can be reversibly precipitated in fibrous form Examined in the electron microscope after staining with PTA the reprecipitated fibrils were found to possess structure the type of which depends upon the conditions of precipitation With increasing ionic strength the band pattern may be that characteristic of native

be shown below the collagen macromolecule very probably has a length of four times the 700 A period i e about 2800 A

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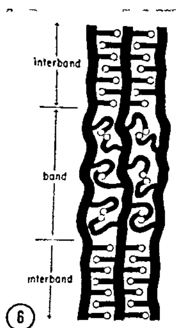


Fig 6 Interpretation of band and interband regions according to Bear (4) Vertical chains represent collagen macromolecules whose side chain interactions produce bands and interbands

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The TC macromolecules are assumed to be essentially identical in structure and composition and to be themselves polarized in the sense of the linear sequence of amino acid residues in the constituent intra molecular strands This is indicated by the arrows on the TC macromolecules in Fig 7 The hypothesis assumes that the various types of ordered patterns of TC aggregation occur by virtue of relatively stable bonding between terminal groups on the side chains of laterally adjacent macromolecules Each type of ordered aggregation type represents a different pattern of interacting side chains In the SLS form it is assumed that the TC macromolecules are essentially in register with respect to their ends and are "pointing" all in the same direction i e they are in parallel array The SLS pattern therefore provides a molecular "finger print" of the collagen hence of amino acid residues along the TC macromolecule and as such is very valuable in further analysis (see below) The FLS is assumed to be formed by an antiparallel packing of TC in which the macromolecular ends are approximately in register (see Fig 7)

Since the axial repeating pattern of native fibrils as seen in electron micrographs and as measured in the small angle X ray pattern is about a quarter that of the length of the TC macromolecules it was assumed that the latter are arranged in parallel array but are displaced in the axial direction by one quarter of a length in adjacent macromolecules (see Fig 7) A specific suggestion somewhat along the same line has been proposed by Tomlin and Worthington (41)

This concept of the structure and properties of the native macromolecule of collagen was deduced from the electron optical observations of the various ordered aggregation types observed The hypothesis received confirmation from the physicochemical studies of Boedtker and Doty (6) performed on solutions which were highly monodisperse with respect to the monomer macromolecules (achieved by centrifuging out the larger aggregates) These data indicated that the macromolecules behave like rigid rods with dimensions about 14×2800 A and molecular weight about 360 000 Previous estimates of other workers about particle sizes were considerably greater probably because their preparations were heterodisperse containing polymers of collagen as well as the monomers

Finally the tropocollagen macromolecules were visualized directly in the electron microscope by a method developed by Hall (18) This consists in depositing the molecules upon the atomically smooth surface of freshly cleaned mica by spraying a very dilute solution of the protein After drying this surface is then shadowed by evaporation of platinum at a small angle The metalized layer is then backed with a

fibrils (period ≅ 700 A) it may be about one third this value or the precipitate may have tactoidal appearance showing no bands at all These different forms can be produced reversibly from acid solutions of highly purified collagen presumably the different ordered states depend only upon the collagen and require no additional organic material

When certain types of extracts are made from connective tissue or when certain organic substances particularly highly negatively charged substances are added to the collagen solutions and the conditions are appropriately adjusted a new modification is formed which manifests an axial repeat or identity period about four times that of normal collagen (i e about 2600–3000 A) and which are therefore called long spacing types Two such forms called fibrous long spacing (FLS) and segment long spacing" (SLS) are shown diagrammatically in Fig 7 The FLS modification is produced routinely by addition of α 1 acid glycoprotein to an acetic acid solution of collagen followed by dialysis against water The SLS modification is routinely produced by addition of ATP to the acid solution of collagen the precipitate forms directly without further adjustment of conditions

It will be noted that the FLS type has a symmetric non polarized band structure while the SLS has an asymmetric polarized pattern of banding

Each of the five band patterns described may be produced reversibly from an acid solution of collagen The particular patterns produced depend for their specificity upon the collagen rather than upon the other substances added or conditions imposed these substances and conditions serve rather to evoke the structures inherently characteristic of the collagen itself

III THE MACROMOLECULAR MONOMER OF COLLAGEN—THE TROPOCOLLAGEN HYPOTHESIS

The above described structures discovered in collaboration with Dr J Gross and Dr J H Highberger were interpreted as follows [see summaries of this work by Schmitt *et al* (37) Schmitt (35) Gross (14) and Highberger (23)] It is assumed that the long spacing (about 2800 A) represents the length of the native collagen macromolecule which has a three stranded helical internal structure as deduced from the large angle X ray pattern (see above) The long thin macromolecules were given the term "tropocollagen" (TC) because they are capable of "turning into" or forming the native collagen structure and also to distinguish them from various other collagen fractions (such as procollagen) which had been previously described

activation before it is capable of being incorporated into fibrous tissue has been much investigated but the details of the process remain to be disclosed

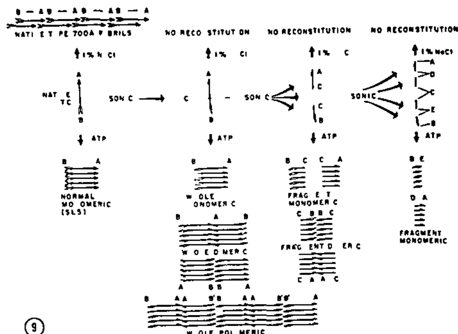


FIG 9 Diagrammatic illustration of the chief effects of sonic irradiation on the tropocollagen macromolecules From Hodge and Schmitt (25)

IV THE FRAGMENTATION OF TROPOCOLLAGEN MACROMOLECULES BY SONIC IRRADIATION

The discovery by Nishihara and Doty (31) that sonic irradiation of tropocollagen rapidly reduces the viscosity without substantial reduction in optical rotation suggested that the irradiation fragments the macromolecules into shorter pieces actually into halves and quarters which retain the triple chain helical structure characteristic of the native macromolecules This possibility was confirmed by Hodge and Schmitt (25) by electron microscopic examination of irradiated collagen The loci along the macromolecules which undergo scission could be determined with considerable precision by reference to the band patterns of the SLS type aggregates produced by the addition of ATP to the acid solutions after irradiation

It was discovered that sonic irradiation produces profound effects

thin collodion supporting film stripped from the mica and examined in the electron microscope at high resolution From such electron micrographs (see Fig 8) Hall (19) found the fibrous particles to be about 15 A in width but their lengths were somewhat smaller than had been predicted by the physicochemical data of Boedtker and Doty (6) Subsequently with improved technique Hall and Doty (20) obtained a weight average length of 2820 A in good agreement with the physicochemical data and with the lengths determined in this laboratory on the same solutions used by Hall and Doty by conversion to the FLS modification and measurement of the axial period (average value was 2700 A)

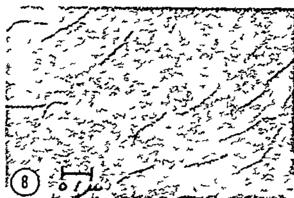


Fig 8 Electron micrograph of tropocollagen macromolecules prepared by the method of Hall (18 19) Courtesy of Dr C E Hall

The problem of the nature of the precursor of collagen in the fibrils of connective tissue has been the subject of much investigation Orekhovitch *et al* (32) suggested that the fraction soluble in citrate buffer ($\Gamma/2 \cong 0.2$ pH $\cong 3.5$) is such a precursor and therefore gave the material the name procollagen However from turnover studies of the incorporation of C^{14} labeled glycine Harkness *et al* (21) suggested that the precursor is to be found in the material soluble in slightly alkaline buffer with a much shorter half life than citrate soluble collagen This conclusion was confirmed by Jackson (28) using other methods The possibility that tropocollagen macromolecules soluble in neutral salt solutions (13) may be the precursor of fibrous collagen has been discussed in some detail by Gross (15) Orekhovitch and Shpikiter (33) have concluded that procollagen and tropocollagen are in fact identical The possibility that collagen as synthesized in the fibroblasts requires

ture in these regions Clues are afforded by a study of the band fine structure in A–A and B–B linkages in the dimeric and polymeric forms As shown in Fig 10 the first bands at the A ends are separated by a region about 100 A long which is a typical interband (i e shows no dense band hence presumably contains relatively few side chains reacting with the phosphotungstic acid "stain") In the case of the B–B junctions however the separation between the first bands is about 180 A and a darkly staining band occurs in the middle of the junctional region



FIG 10 Whole polymeric aggregation types of SLS aggregates from a solution of calfskin collagen treated with sonic irradiation for 240 minutes Locus of A and B ends of macromolecules labeled Arrow points to dense band at the junction between macromolecules at B end From Hodge and Schmitt (25)

This behavior is highly suggestive concerning the nature of macro molecular ends and of end to end polymerization of macromolecules as follows (*1*) chain appendages may occur at both ends of the native TC macromolecule (*2*) these appendages may have lengths of about 100 A and 200 A at the A and B ends respectively (*3*) the amino acid composition of the terminal chain appendages resembles typical inter band regions i e lacking concentrations of basic amino acid side chains thought to characterize the band regions (except for a portion of the B end as mentioned above)

in addition to scission of the macromolecules into smaller fragments The most striking of these is an alteration of "end regions" produced by relatively short periods of irradiation without change in the length of the macromolecules The results are shown schematically in Fig 9 wherein the native TC macromolecules are represented by an arrow with A and B ends indicative of the asymmetric distribution of amino acid residues reflected in the SLS type of aggregation pattern It is thus possible at a glance to tell which is the A and B end of any particular SLS in addition to the specific band pattern at each end the polarization of the TC is shown at once by the position of the broad slightly off center interband [labeled F G by Schmitt *et al* (37)] As was previously indicated the formation of the native type (700 A axial repeat) involves the formation of protofibrils that are actually linear polymers of TC by end to end interaction of the A–B type lateral aggregation of such protofibrils occurs in a manner such that adjacent protofibrils are displaced axially with respect to one another by a quarter of a macromolecular length (about 700 A) It is this A–B type of interaction of macromolecular ends that is first affected by sonic irradiation In the diagrammatic representation the altered ends are designated A and B

Following irradiation sufficient to prevent the formation of native type fibrils (tested for by dialysis vs 1% NaCl) i e by alteration of macromolecular ends two pronounced changes are found in the ATP precipitates (*1*) an increased side to side interaction producing a highly exaggerated lateral aggregation into long ribbons of SLS forms and (2) a progressive increase in the amount of A′–A and B–B type interaction As a result the formation of dimeric and polymeric forms is favored (see Fig 10) With longer irradiation the macromolecules are fragmented the locus of the scission being indicated by the band pattern of the fragments

It is noteworthy that end to end polymerization of scission products never involves ends produced by the fragmentation (such as those designated C D or E in Fig 9) Apparently the original ends of macromolecules are different from those produced by sonic scission From the high density of bands (i e regions which combine preferentially with phosphotungstic acid) in end regions of SLS it seems clear that certain amino acid side chains (possibly the guanidino groups of arginine as suggested by Kuhn *et al* (29) or the epsilon amino groups of lysine) may be concentrated in the end regions of the macromolecule

Because of the special properties of the end regions in end to end polymerization it is important to obtain evidence concerning the struc

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Discussion

Dr Sherry Can you solubilize all kinds of collagen? We could solubilize rat tail collagen but not Achilles heel collagen

Chairman Schmitt That is correct

Dr Sherry Do you have any data on the mechanism of action of the clostridial collagenase?

D S Jackson (26) failed to detect any peptide formation during the precipitation of neutral salt extracts It may be that the end effects discovered by Hodge and Schmitt (25) in their experiments with sonic irradiation of TC will provide some clues in this problem Their technique provides a sensitive means of investigating the normal A–B type of TC polymerization as well as abnormal A–A′ or B–B types of interaction Using these techniques experiments are now in progress in which the specific nature of the "end reaction" of normal polymerization enzymatic and non enzymatic is being investigated

One of the most striking lessons taught by investigations of complex biological problems such as those of collagen briefly reviewed in this paper is the importance of isolating and characterizing as nearly as possible at the molecular level the individual components involved This is particularly true where elongate macromolecules are involved because it is becoming clear that in such processes the interaction of the macromolecule with others of its own or of another kind may be highly specific and may in appropriate chemical environment lead to higher levels of structural or functional organization Pathological processes may also have their origin in such interactions Clearly one is in a better position to investigate such processes after the molecular actors in the play have been identified and at least partially characterized The great theoretical and instrumental advances in the physicochemical investigation of macromolecular systems made in recent years have provided the means for such advances It remains only to apply these means in carefully planned programs of research in the biomedical sciences

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ably not have been associated with collagen at all because as everyone thought a few years ago the molecular fingerprint of collagen has a length of 640–700 A If FLS or SLS are at some future time discovered in tissue preparations we would immediately investigate the nature of the noncollagenous substance that is combining with the tropocollagen macromolecules to produce the long spacing modification Even if such a discovery were possible only in frankly pathological situations one need not think of the collagen (macromolecules) as being "pathological" Rather one would seek to determine the conditions in the pathological tissue which caused the tropocollagen macromolecules to aggregate in abnormal manner

DR DUGUID Is it possible then to have a collagen fiber in one state lying immediately next to another collagen fiber in an obviously different state?

CHAIRMAN SCHMITT Entirely possible We have electron micrographs which show side by side normal 640 A. collagen with fibrous long spacing type In fact if one chooses the concentration of glycoprotein correctly one can by adding the glycoprotein to an acid solution of collagen and dialyzing produce at will precipitates which contain mixtures of 640 A and FLS type fibrils seen side by side on the electron micrographs

DR MEYER Are the amino acid sequences well enough known so that you can predict either by hydrogen bonding or polar bonding whether they are or are not identical especially since you don t know whether the three strands have a single helix?

CHAIRMAN SCHMITT It is necessary before attempting to do a step wise analysis of amino acid sequence to separate the individual covalent strands and collect them in separate fractions Progress is being made in this direction in several laboratories

DR SMITH When the three chains are separated are they different?

DR MEYER You cannot separate them

CHAIRMAN SCHMITT They can be separated but there is still much question about the status of the strands before separation

DR. MEYER But can you separate each of the three strands? Let us say you have to have blue green and red You can only have red ones blue ones or green ones or you always have a mixture of the three So far as I know this has not been done

DR. SMITH Did you separate the blue and green and red ones as to their amino acid composition?

CHAIRMAN SCHMITT No This whole business is only a few years old and conclusions must still be very tentative Originally Doty claimed one chain was 120 000 and another about twice that weight He thought an ester bond involving serine made a double length chain which by folding in the middle could in the helix behave as two chains A pH of about 10–12 is required to break the ester bond

Doty has clearly shown, as has Orekhovitch in Russia that physicochemical evidence can be obtained about the size of th se chains Grassmann s highly competent analyses of amino acid composition and sequences are made after heat denaturation followed by tryptic digestion These and other studies such as those of Schroeder at California Institute of Technology and Kroner of the United Shoe Machinery Corporation cannot be meaningful concerning the larger helix structure of the macromolecule until the individual covalent chains are isolated and fractionated. If one wanted to maintain that there are only two chains or possibly only one chain he would have to show what kind of linkages cross-bond the chains or chain at the folds that permit the formation of a three stranded helical configuration

CHAIRMAN SCHMITT No I do not Dr Paul Gallop and others have been working on that

DR WHITE Dr Gallop and Dr Sam Seifter have purified the collagenase from *Clostridium histolyticum* In the early stages of incubation of neutral solutions of different collagens with collagenase the viscosity rapidly goes down and the ninhydrin values increase while the optical rotation remains relatively high They have concluded that the enzyme initially causes scission of one or two strands of the collagen molecule without seriously disrupting the helical structure thus explaining the viscosity and rotation data

DR SHERRY Has Dr Gallop studied papain in acid solution?

DR WHITE No The collagenase which they have has no proteolytic activity against a variety of other proteins

CHAIRMAN SCHMITT Dr Gallop found that it does produce scission of chains in many other places

DR DUGUID After staining an amorphous material is mixed up with the collagen fibers but amongst them are sometimes fibers of a finer character with a 230 A periodicity

CHAIRMAN SCHMITT The 230 A period is one third of the 700 A period and it often turns up in electron micrographs Keith Porter originally suggested that in their biogenesis as studied in tissue culture collagen molecules 210–230 A long are formed first During development these are transformed to molecules having the standard period of 640 A However it is now apparent that like the 640 A period that of 210–230 A may be produced by a particular axial displacement of the tropocollagen macromolecules and is brought out by a particular method of preparation

What is pathological collagen? How would one even know what collagen is unless one purified it and determined its structural and chemical properties as a molecule or macromolecule? Can we say that a collagen fibril that looks a little different or stains differently is pathological ? Certainly not until we have explored the full range of states possible for normal purified collagen

DR DUGUID If I produce a fibril at 230 A is that normal collagen?

CHAIRMAN SCHMITT Yes it might very well be Gross Highberger and I demonstrated that it could be produced reversibly from purified collagen preparations which were also capable of yielding all the other forms of collagen

DR DUGUID Well then how do you attack this problem? Is it worth electron microscoping pathological material? How can you isolate collagen?

CHAIRMAN SCHMITT One might very well be led astray if he drew conclusions about collagen in pathological material before the facts are established regarding normal collagen One must find out as much as possible about the native molecule itself and how it is subject to variations due to environment Collagen behavior depends upon its built in structural and chemical specificity and that in turn depends on the specific amino acid array in each covalent chain and on the way these chains are aggregated to form the three stranded macromolecule How the built in specificity is manifested depends on the chemical environment in which the macromolecules find themselves at the time

The long spacing modifications of collagen structure are an interesting case in point There have been a few reports of observations of FLS in tissue preparations but so far as we know neither FLS nor SLS is stable in normal tissue possibly because of the relatively high ionic strength prevailing there Now if either FLS or SLS had been first discovered in some pathological preparation it would prob

were three other possibilities depending on whether the individual chains are identical

DR JACKSON I still do not see how you account for the different types of aggregation in terms of those dangling ends I can see the long spacing thing resulting from hooking up two of the dangling ends in parallel giving two molecules

CHAIRMAN SCHMITT Or polymers

DR JACKSON Yes How do you account for the quarter overlap to give you the 640 A spacing and still implicate the dangling ends?

CHAIRMAN SCHMITT Monomers and interaction regions would be staggered to form the 640 A period Any explanation of this type of aggregation requires the fit to be precise and this in turn requires that the end to end union of TC monomers be precisely and essentially uniquely accomplished This is in fact demonstrated in the band patterns of the dimeric and polymeric forms described by Hodge and me in the sonically treated material The coiling of end chains in adjacent monomers proposed by Hodge and me would provide such precise union

DR MEYER You picture the effect of the prolonged sonification as breaking peptide bonds?

CHAIRMAN SCHMITT Certainly This is demonstrated by the electron micrographs of the ATP precipitated SLS forms

DR SMITH Can you pick up the end groups?

CHAIRMAN SCHMITT This has not yet been attempted

DR JACKSON I think he wants to do the end group analysis If you assume the peptide is broken then you must assume the release of end groups

DR SMITH I am not satisfied that the structure of collagen in terms of three strands is right if this were purely polypeptide material because one should have a free amino group and a free carboxyl group Nobody can detect those

CHAIRMAN SCHMITT Collagen is not the only protein that has that trouble

DR SMITH No but you should again detect three new amino groups and three new carboxyl groups stoichiometrically This has yet to be determined or proved

CHAIRMAN SCHMITT These experiments are very recent and the experiments you suggest and which would be very welcome have not yet been done

DR SMITH You do not have to fractionate to find out the number of new end groups

DR GITLIN He has more than one period broken here roughly into halves and quarters It could be anywhere along those lengths Theoretically you should be able to fractionate it

CHAIRMAN SCHMITT We would be glad to furnish the material for you to do it

DR MOON Is there evidence that in the human body under any abnormal condition collagen is formed other than the 640 A type?

CHAIRMAN SCHMITT No The 640 or 700 A is quite characteristic of the collagen class of proteins However as was previously pointed out *the failure to demonstrate* any periodicity in a given fibril does not prove that it is not composed of undenatured native collagen The packing of the macromolecules may be such as to prevent the formation of the typical 640 A pattern or it may lead to quite different patterns some of which may still remain to be discovered

DR MEYER But one would not expect to get transpeptidation in this way None of these procedures would change the amino acid sequence

CHAIRMAN SCHMITT That is correct

DR MEYER From the published data of Grassmann you cannot explain Crick s model

CHAIRMAN SCHMITT That may be true but it does not in itself disprove the Crick Rich model One of the interesting discoveries of the Grassmann group is the discovery of certain peptides that are either highly basic or highly acidic If these are located at specific regions in the macromolecule they might explain certain of the properties of TC This assumes that artificial recombination of hydrolytic products did not occur under the conditions of Grassman s experiments

DR SMITH Aside from that if there are three simple polypeptide chains why is there no end group?

CHAIRMAN SCHMITT The peptide linkage mentioned by Doty and by Grassmann might explain the failure to find two of the possible six ends

DR JEANLOZ Is what you call a molecule with three strands one molecule or three molecules?

CHAIRMAN SCHMITT There are two and possibly three molecules because if collagen is put in urea or guanidine hydrochloride the individual ones are freed and can be characterized by physicochemical means

DR JEANLOZ Is one of these parts just one of the strands?

CHAIRMAN SCHMITT It is difficult to fractionate the individual chains

DR JEANLOZ Would the macromolecule be in reality three molecules twisted together?

CHAIRMAN SCHMITT Yes That is why I call it a macromolecule because the bonds holding these molecules together are hydrogen bonds No end groups were found in a number of other proteins which had clearly fibrous structure The crystallographic data seem fairly clear about the way in which the helix is arranged

DR SMITH The spacings don t fit exactly

CHAIRMAN SCHMITT The complete data and computations have not yet been published by Crick and Rich but it is perhaps fair to say that no basic inconsistencies have as yet been found with the model and that it is the only current model about which this can be said The helical configuration can hardly be questioned though the detailed composition and bonding of the covalent chains is still far from understood

DR MEYER He can explain roughly one quarter of the molecule

CHAIRMAN SCHMITT Isn t it more nearly the other way one quarter of the molecule may not have the three stranded helical structure?

DR JEANLOZ On what basis do you consider two different modes of action of the sonification? One would change the aggregation the other would break the chain Why not consider only one action?

CHAIRMAN SCHMITT I said there seemed to be two different modes of action that can be distinguished by their rate constants There is a fast reaction that does not break the molecules because all the reconstituted material is of normal length This rapid process results in failure of the reprecipitated material to show the slower process causes scission of the molecules

DR JACKSON You showed the molecules with dangling ends You also said earlier you got overlapping

CHAIRMAN SCHMITT Yes Three or four years ago the Dotys suggested there

Chemistry of the Fibrous Elements of Connective Tissue*

DAVID S JACKSON

Department of Surgery Harvard Medical School and The Fifth Surgical Service and Sears Surgical Laboratory Boston City Hospital Boston Massachusetts

The physicochemical properties of collagen and some of its biological properties have already been dealt with by Dr Schmitt and Dr Gross The object of this paper will therefore be to review briefly the chemistry of collagen with particular reference to the various collagen fractions which have been discovered in recent years An attempt will also be made to clarify the biological significance of these fractions and their place in the over all pattern of fibrogenesis

The chemistry of reticulin and elastin, the other major components of connective tissue will also be considered although in less detail than collagen Extensive reviews of the physicochemical properties of collagen are available (6 8) and the biochemistry has also been reviewed by Jackson (16) Therefore only a few references will be given

I COLLAGEN

The amino acid analysis has been very thoroughly worked out and provides an unusual pattern Hydroxyproline which occurs uniquely in collagen (with the possible exception of elastin) and proline make up almost 30% of the total while glycine accounts for a further 33% Aromatic amino acids are present in low concentration and cysteine is completely absent Hydroxylysine again an amino acid unique to collagen is present in low concentration (1 2%)

II THE SOLUBLE FRACTIONS

Collagen is normally an extremely insoluble protein rendered soluble only by autoclaving to form gelatin which although very similar chemically to collagen has lost the unique structural configuration of the parent fibrous protein But since 1900 when Zachariades showed that rat tail tendon was partially soluble in dilute organic acids there has been a spasmodic interest in the possibility of the collagen fibres having a soluble precursor Nageotte (18) showed that protein extracted

* This investigation was supported in part by a research grant (A 1867) from the National Institute of Arthritis and Metabolic Diseases Public Health Service

activities of which vary considerably At 8 hours the highest specific activity is found in the fraction extracted with 0 14 M NaCl the specific activity decreasing with increasing salt concentration The situation at 24 hours after injection of the isotope has been almost completely reversed the most active fraction being that extracted with 1 M NaCl Harkness *et al* (11) have previously shown a similar gradation of specific activity in several extracts of rabbit skin with citrate buffer at pH 3 6

Thus it would seem that following the synthesis of the collagen molecule increasingly larger and more firmly cross linked aggregates of these molecules are formed requiring more and more drastic methods of extraction At any given time in growing connective tissue there will exist a continuous series of such aggregates The fraction of these extracted will thus depend on the salt concentration and the pH of the solution The indications are that there is no single *extracellular* precursor although that extracted with 0 14 M NaCl is the most recently synthesized and may include some of the intracellular collagen molecules

III Insoluble Collagen

With time the strength of aggregation will increase as thermal agitation allows the constituent molecules to fit into their best crystallographic arrangement As a newly formed fibril develops the original fibril will accumulate further collagen molecules thus increasing in length and thickness Because of the aging phenomenon described above the developing fiber will also be heterogeneous since the inner core laid down first will have reached a higher degree of aggregation than the more recently laid down outer layers This heterogeneity will continue until the whole fiber has ceased to grow and the aging process is complete throughout the fiber

This will explain the collastromin of Tustanovskii *et al* (29) and the metacollagen of Banga *et al* (1) These workers believe that the collagen fiber consists of two fractions the procollagen of Orekhovitch (19) and an insoluble core properties of the fiber depending on the presence of both However both collastromin and metacollagen are the result of a combination of acid pH and heat which will lead to partial degradation of the constituent collagen molecules which is irreversible causing an alteration in their structural integrity These two fractions cannot therefore be considered to have any biological significance

IV The Synthesis of the Collagen Molecule

The problem of the synthesis of the collagen molecule has all the features in common with the over all problem of protein synthesis with

from rat tail tendon with dilute acetic acid could be precipitated in a fibrous form having all the tinctorial properties of native collagen fibers Later this material was shown to be identical with collagen when examined under the electron microscope and by X ray diffraction (26 31) and also in its amino acid composition

Later in 1947 using acid citrate buffers Orekhovitch (1952) isolated a collagen like protein from several connective tissues and named it procollagen A further fraction was isolated from extracts of connective tissue with neutral or slightly alkaline salt solutions and was named neutral salt soluble collagen (5 11 12 13) A wide range of salt concentrations has been used to extract the latter

Considerable confusion has arisen concerning the significance of all the soluble collagen fractions

The salient point about all these fractions is however that once isolated and purified they are all identical in their physicochemical properties and are interconvertible (6) In solution under the same conditions all behave as elongated rigid rods having the dimensions of approximately 3000 A by 15 A and having a molecular weight of about 340 000 (6)

If these fractions have any significance it must be sought in their occurrence in the biological system

Both Nageotte and Orekhovitch believed that the acid extracted fraction was the soluble precursor of collagen fibers The proportion of this fraction in connective tissue decreases with age the uptake of C^{14} glycine into this fraction is higher than that in the insoluble residue and this uptake is considerably reduced in ascorbic acid deficiency a condition known to inhibit collagen formation However Harkness *et al* (11) demonstrated an even higher rate of incorporation of C^{14} glycine into the neutral salt soluble fraction and this was confirmed in a study of the formation of collagen in the carrageenin granuloma (15) Further Gross showed that neutral salt soluble collagen disappears very rapidly from the skins of guinea pigs on a protein free diet or on a scorbutogenic diet It would thus appear that neutral salt soluble collagen is the precursor of collagen fibers and Gross (7) has shown that merely warming a solution of collagen at neutral pH results eventually in the formation of insoluble collagen fibers

It is likely however that neutral salt soluble collagen is itself biologically heterogeneous Salt concentrations ranging from 0 14 M to 2 M have been used to extract this fraction with considerable variation in the amount of collagen extracted Recent studies in my laboratory have confirmed this heterogeneity Several extractions of skin and carrageenin granuloma from guinea pigs which have received a single injection of C^{14} glycine 8 and 24 hours prior to death yield fractions the specific

having the characteristic pattern of collagen and at least two hexoses This latter type of reticulin never develops further into the typical col lagen fiber in normal physiological circumstances

VI Elastin

Chemical and biochemical studies on this fibrous protein have devel oped much more slowly than studies of collagen with which it almost always appears closely associated [for review see Hall (10)]

Elastin gives an ill defined X ray pattern which Astbury tentatively assigned to the collagen group Examined under the electron microscope it appears as a fibrous or sheet like structure having no recognizable periodicity It is extremely difficult to purify from the closely associated collagen The amino acid analysis of highly purified elastin from liga mentum nuchae has been carried out by Partridge and Davis (21) The amino acid pattern shows an unusually high concentration of non polar amino acids which compares well with the non polar nature of rubber and gives elastin similar physical properties of reversible extensibility and low degree of swelling in aqueous solvents Partridge *et al* (20) have shown that a short hydrolysis of purified elastin with oxalic acid gives rise to two components α-elastin which has a high molecular weight (around 60 000–80 000) and β elastin which is a polypeptide with a molecular weight of about 6000 α Elastin has the property of forming a type of gel under various conditions of temperature and pH and Wood (30a) has shown that this gel can become insoluble when gelling conditions are prolonged

Partridge and Davis (21) have suggested that elastin is chemically homogeneous and consists of a disordered structure composed of ran domly coiled chains lying generally parallel to the fiber axis The high degree of insolubility and low degree of swelling suggests the presence of strong cross linkages The nature of these linkages remains in doubt The physical properties of the elastin fiber may be due to inhomogeneity at a higher level of organization This is suggested by the work of Hall and his associates (9) who suggest that elastin should be considered as two phases one amorphous which surrounds a fibrous phase and affords protection against solubilization This outer phase was considered to contain mucoprotein which is removed by one of the components of elastase leading to the spontaneous dissolution of the central core In view of the findings of Partridge *et al* the hypothesis is somewhat con troversial since this group finds negligible amounts of hexose and hexo- samines in their preparations

Even more controversial is the hypothesis put forward by Burton *et*

the addition of a complicating factor That is the fact that the two amino acids unique to collagen hydroxyproline and hydroxylysine can not apparently be incorporated directly into the molecule but must go by way of proline and lysine respectively which are hydroxylated at some stage of the synthetic process Stettin (29) suggested that the hydroxylation of proline took place at the protein or peptide stage and a similar mechanism was suggested for hydroxylysine (23 27) A col lagen like precursor lacking hydroxyproline has been postulated and evidence for its existence in the connective tissues of scorbutic guinea pigs has been put forward (2 25) The former authors isolated a pro tein from the carrageenin granuloma following gelatinization which appeared to have a low hydroxyproline However the present writer was able to fractionate this into normal gelatin and a fraction contain ing no hydroxyproline and a concentration of proline lower than that of collagen instead of the expected higher concentration (Jackson unpub lished data) Robertson (personal communication) has recently pro duced evidence making his earlier hypothesis untenable Moreover Green and Lowther (4) have obtained ratios of specific activity of hy droxyproline to proline following incubation of granuloma slice with C^{14} proline greater than 1 a finding incompatible with the Stettin hy pothesis It is possible since free C^{14} hydroxyproline can be identified following incubation with C^{14} proline (4) that the formation of pro tein bound hydroxyproline proceeds by way of "activated proline which is hydroxylated to form "activated hydroxyproline which is then in corporated into the collagen molecule hydroxyproline cannot be acti vated directly hence the inability of exogenous hydroxyproline to be incorporated into collagen directly

V Reticulin

This subject has been well reviewed by Robb Smith (24) Briefly there appears to be at least two types of reticulin One occurs in de veloping connective tissue preceding the thicker collagen fibers These appear to be thin collagen fibers soluble in acid citrate buffer whose silver staining can be abolished by extraction with neutral salt solutions (14)

The other type is that associated with the basement membranes of the parenchymatous organs between connective tissue and epithelium and around muscle and nerve fibers The reticulin from at least one of these sources appears to be an extremely insoluble complex of collagen lipid and a polysaccharide (30) which can only be dissociated by com plete acid hydrolysis when it gives rise to myristic acid amino acids

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Discussion

Dr White There might be an analogy in the iodination of aromatic nuclei present in intact proteins If one can jump from iodination to hydroxylation then one might have hydroxylation of peptide bound proline

Dr Jackson I do not suggest this i correct except that the evidence favors it and one does not have to assume peptide hydroxylation One can explain the facts by this system instead of the other Almo t certainly some is bound to be unstable and some break down into free hydroxyproline

Gold has suggested in vitamin C deficiency the failure is that of proline to hydroxylate A lesser hypothesis is that one would expect to find a protein none of which has been hydroxylated Dr Gross and I have looked at skin fractions of the carrageenin granuloma in C deficiency a d nowhere can we find anything re motely resembling this Robinson in Vermont reports that he found a gelatin with little hydroxyproline but I fractionated that into normal gelatin having the right amount of hydroxyproline

Dr Boucek Wh t were Neuberger s riteria for the identification of free labeled hydroxyproline? Was it dialyzable?

Dr Jackson You can actually isolate the free hydroxyproline without prior treatment at the same time that you isolate the collagen fraction

Chairman Schmitt That is rather fortunate because it would be very difficult to get the triple helix to form without the hydroxyproline

Dr Hartroft You always started off from fibroblasts in the formation of col lagen In cirrhosis particularly experimental cirrhosis there is much laying down of collagen but some of us have difficulty in identifying first class fibroblasts Can

al (9a) and supported by detailed electron microscope studies by Keech (17) that there exists a range of proteins intermediate in composition between collagen and elastin and that collagen can be converted *in vitro* into elastin by a wide range of chemical and enzymatic reactions This hypothesis has been severely criticized by Partridge (22) and in the opinion of the present writer considerably more chemical evidence is required before it can be accepted There are many difficulties in this hypothesis a major one being that to obtain the correct amino acid pattern for elastin would involve splitting the collagen molecule into very small peptides to retain the proline while removing the hydroxy proline since the sequence gly pro hypro gly is an essential structural element of crystalline parts of the collagen fibril (3)

VII Cellulose

Until recently the fibrous elements of mammalian connective tissue were limited to collagen reticulin and elastin but recent work (10a) has indicated the presence of cellulose in normal human dermis The cellulose appears to be oriented on a core of protein whose amino acid composition is suggestive of collagen It would seem that cellulose will now have to be considered as one of the fibrous elements of connective tissue

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tradict the hypothesis that reticulin can be transformed into collagen?

DR JACKSON The final form of the collagen fiber depends almost entirely on the environment in which the fiber is being formed In the vitreous humor of the eye at ultramicroscopic levels you see an extremely fine meshwork of collagen fibers exactly like that produced if one gels neutral collagen in fact Dr Fessler in Dr Gross s laboratory has made what might be called synthetic gel, by gelling collagen in the presence of hyaluronic acid showing improved water binding capacity

DR HARTROFT In certain states of destruction of parenchyma of the liver the reticulum is squeezed together and some think this could be transformed into what we call collagen

DR JACKSON It is just possible that either the preformed reticulum does thicken or whatever process is going on actually destroys the original reticulum replacing it with fibrosis and it goes on to normal thick collagen fibers

DR LANSING May I just pass on to Dr Jackson the information that the uterine artery work was done by Dr Arthur Schwartz in the early twenties and appeared in the *American Journal of Obstetrics and Gynecology*

DR AMBRUS I wonder how all this information could be used to answer Dr Duguid s questions Perhaps he is better off trying to identify the nature of these fibers by ordinary enzymatic histochemical methods such as using collagenase elastase fibrinolysin and maybe even cellulase rather than the electron microscope

CHAIRMAN SCHMITT It is quite possible that treatment of a specimen with specific enzymes such as highly purified collagenase would give useful information to the pathologist as to the location of collagen However until one understands how the normal collagen fiber is formed presumably by a possibly complex process from the precursor native macromolecules it would be difficult indeed to attack the problem of the nature of collagen fibers in pathological states One would have to establish that the pathological process has anything to do with the fibril itself rather than with the processes which favor the formation of fibrils from dissolved macromolecules or prevent their disintegration under special conditions

DR WHITE We may not have pathological collagen The pathology of collagen may only be in the quantity rather than the quality

DR. DUGUID Dr Jackson you are assuming that the material that you are working with in the carrageenin granuloma is collagen are you not?

DR JACKSON I relied almost exclusively on my own work for this point of view I should have brought in Dr Gross work on normal guinea pig and other animal dermis which leads one to the almost identical conclusion

I can isolate and identify collagen chemically and electron microscopically It is a misnomer to call this a fibril as some do because there is more lipid than collagen formed in this situation I make certain that what I get out is collagen alone by chemical and other methods so I can safely say this is what is happening to collagen in the carrageenin granuloma

I would not claim this is a normal situation Within 6 weeks of producing a carrageenin granuloma in the guinea pig it disappears This in itself is interesting because at the moment I am more interested in what happens when collagen is absorbed than when it is being built up One can show that it melts away by forming soluble collagen The longer it goes on the higher the concentration of soluble products and these can be identified as neutral collagen

DR DUGUID In any inflammatory process including the carrageenin granuloma fibrin comes into the process at one stage We do not know what happens to the fibrin It may be picked up by cells and carried away and something else put

you envision a situation where this could happen without fibroblast or do you think the fibroblast always has to be found?

DR JACKSON The fibroblasts by definition are cells that produce collagen fiber Is not the earthworm the one situation where the epidermal cell produces collagen?

CHAIRMAN SCHMITT However this is a very unusual collagen It has 40% more hydroxyproline than mammalian collagen and much less proline It also lacks in the longer side chains which it is believed are involved in the formation of bands and *interbands as seen in the electron microscope This would seem to offer a pos* sible reason of why one sees no band pattern in earthworm fibrils although the material does show the typical collagen large angle pattern (which of course re sults from the internal architecture of the collagen macromolecules)

DR LANSING Dr Jackson why do you apply this definition to the fibroblast presumably a collagen producing cell and not do the same for the elastic fiber? Why do you object to the existence of an elastoblast as I gather you did but you are quite willing to accept the fibroblast?

DR JACKSON I do not object to the existence of the elastoblast I know of no evidence for the existence of such a cell If you cultivate fibroblasts in tissue cul ture they produce nothing but collagen They do not produce elastin

DR LANSING Around 1930 Vance de Coverley worked with tissue culture and described fibroblast like cells with the typical indented nucleus whose cytoplasm reacted quite specifically with orcein He recognized elastic fibers Hobbs showed in the postpartum uterine artery during involution of the uterus that there are cells that look like fibroblasts which line up in parade and adjacent to them one finds the deposition of a new elastica interna This I think would fit the requirements you give us

DR JACKSON This is the first time I have known any work that was satisfying on this point

DR LANSING I think the fibroblast in relation to the collagen fiber is just as nebulous or the evidence of its function is just as tenuous as the elastoblast rela *tionship to the elastic fiber*

DR JACKSON No I would not accept that at all The Fitton Jackson work using thin sections of embryonic tendon and embryonic bone where one can see the fibroblasts or the osteoblasts shows collagen fibrils forming on the surface of the cells and moving out into extracellular space Then they begin to aggregate the fibers growing thicker probably because this fibroblast is still secreting the collagen The cells are readily identified in the sections as fibroblasts

DR MEYER From the literature on tissue culture and regeneration it seems to me convincing that there is a connective tissue cell which produces elastic fibers

DR JACKSON *I agree All I said was I did not know any evidence for the* identification of such a cell

One has this almost three dimensional lattice work of randomly coiled chains which are highly organized in the sense that the helix is not highly organized which is already fully extended The thing is already in an unextended form

DR MEYER Does it become oriented in X ray diffraction?

DR JACKSON Yes but if one stretches it and draws it it will not return to its original length It stays put

DR MEYER No but if you wet elastic fiber or stretch it—

DR JACKSON You get a better X ray picture It does not give a collagen picture

DR HARTROFT Is the fibroblast concerned with the production of reticulin just as it is with collagen? Under pathological conditions is there anything to con

On the Significance of the Soluble Collagens*

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The old observations of Laplat, Nageotte Faure Fremiet, and others on the partial solubility of collagen in dilute organic acids have provided a tool in recent years for the study of the molecular structure and metabolism of this protein F O Schmitt has discussed at this symposium our earlier studies on the reconstitution of collagen from acid solutions from which we derived the concept and partial characterization of "tropocollagen" the molecular building block of the collagen fibrils (5 29) He has also discussed his more recent studies on the interactions between certain breakdown products of tropocollagen (19)

In 1948 and succeeding years Orekhovitch and colleagues (26 28) described the extraction of collagen from a variety of vertebrate connective tissues with organic acid buffers such as 0 1 M citrate pH 3.5 This extraction differed from the earlier acid extracts in that Na or K ions replaced some of the H and the ionic strength was higher Neutralization of these extracts produced "crystalline" fibers which the Russian workers termed "procollagen" We examined similar preparations in the electron microscope and found many of these fibrils to have the typical axial periodic structure of the native fibril and the remainder to be of the "long spacing" type described elsewhere (17 18)

Orekhovitch and colleagues found the amount of "procollagen" to be diminished in advancing age and in scurvy (26 27) They also observed greater and earlier incorporation of labeled amino acid and Deuterium in "procollagen" than in the insoluble residue and concluded that the citrate extractable fraction was the precursor of the insoluble collagen

In 1951 (18) we described the presence of a form of collagen ex

This is publication #238 of the Robert W Lovett Memorial Foundation for the Study of Crippling Disease Harvard Medical School at the Massachusetts General Hospital Some of the work described herein was supported by Grant #A90 C(5–8) of the National Institute of Arthritis and Metabolic Diseases United States Public Health Service and accomplished during the tenure of an Established Investigatorship of the American Heart Association

down in its place There is no doubt that in a mass of fibrin collagen fibrils appear after a while I do not know whether they appear by transformation of the fibrin or are manufactured by fibroblasts but I think we are on dangerous ground if we assume a tissue that has been produced by a pathological process such as inflammation is collagen There are several products of the inflammatory reaction including fibrin and I think we are unsafe to address ourselves to one product and ignore the rest

You said any amount of collagen can be produced in tissue culture But can it? I have never yet seen the tissue culture in which there were great bands of fibrous tissue a hundred or two hundred times the size of the cells as we see it in scar tissue for example

CHAIRMAN SCHMITT Earl said he would present me with 25 gm of it if I so desired

DR DUGUID Yes but it takes an awful lot of tissue culture to produce 25 gm

CHAIRMAN SCHMITT I would like to make a few observations about fibrin According to Astbury's early work fibrin is in a different crystallographic class from collagen The former a member of the so-called KMEF class is presumably an alpha helix while collagen is presumably a triple helix It is difficult to see how one could be directly transformed into the other as was claimed by Baitsell In addition of course the band pattern of collagen and fibrin are very different as seen in the electron microscope Collagen has an axial repeat pattern of 600–700 A with a very specific pattern of intraperiod bands while fibrin fibrils when appropriately prepared have a band structure with an axial repeat of 235 A This distinction between collagen and fibrin is an excellent example of the value of the biophysical approach even apart from the differences in chemical composition which can be demonstrated between the two substances

salt extracted collagen in dilute acids and acid extractable collagen in cold neutral salt solutions With regard to solubility properties the structure of the fibrils they form and their physical chemical characteristics no significant differences have been observed between them (2 14) Studies on amino acid composition (23) have revealed certain small variations between acid and neutral salt extractable and insoluble

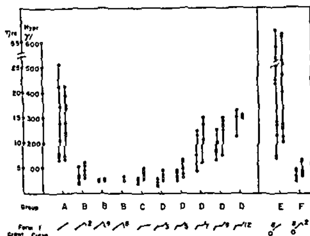


FIG 1 The effect of growth rate on the cold neutral salt (0 45 M NaCl) extractable collagen of guinea pig dermis Each point represents one animal ● = relative viscosity of extract (which depends on collagen content) ○ = hydroxyproline content of the extract The symbols shown on the figure for the form of the growth curves represent the controlled pattern of growth manipulated by feeding Group A = normally growing animals the three B groups = animals starved for 2 4 and 6 days after 10 days of normal weight gain Group C = weight kept constant for 14 days by restricted diet after period of normal weight gain the five D groups = animals treated same as previously except that restricted animals were fed *ad lib* for various time periods (indicated by number in lower part of figure directly below the group) and actively regained weight Group E and F = animals charted from birth to 8 days when killed group F was starved for an additional two days before processing From Gross (10)

collagens but these may be attributable to small amounts of contaminants There is of course the possibility that the very earliest precursor molecules which may be called collagen (in that they have the full complement of amino acids in the correct structural relationships) may not be capable of fibril formation either because the structural organization of the molecule is not quite complete or because of the presence of an attached peptide or other grouping which inhibits association and awaits

tractable from fresh connective tissue in cold slightly alkaline salt solutions and in 1955 we (6) and also Jackson and Fessler (20) reported the extractions of collagen in cold neutral salt solutions at hypertonic and physiological ionic strengths Gross (9) has recently described some of the characteristics of these extracts in more detail

Harkness *et al* (16) in 1954 studied the incorporation of C^{14} labeled glycine into the alkaline and citrate extractable collagens and insoluble residue of rabbit skin demonstrating the most rapid uptake in the alkaline fraction which also had a half life of about 48 hours The citrate extractable fraction showed a much slower incorporation rate and a curve of specific activity from which a half life could not be estimated The insoluble collagen had a negligible uptake of isotope In 1956 D S Jackson (21) reported similar results using incorporation of C^{14} glycine into the collagen of the carrageenin granuloma in guinea pigs The fraction extracted in cold 0 2 *M* NaCl had a half life of about 16 hours with a relatively high specific activity and the citrate extractable fraction (procollagen) and insoluble residue as in the earlier study on rabbit skin (16) showed slow rates of incorporation and much lower maxima These two investigations provided strong evidence indicating that the alkaline and neutral extractable collagens were very recently synthesized (with respect to the time of their extraction) as compared with the acid extractable collagen The latter in turn may have been more recently synthesized than the insoluble residue Further evidence on this point was obtained by the writer (10) in an investigation of the effect of growth rate on the neutral salt extractable collagen in skin In actively growing 8 day old guinea pigs this collagen fraction (extracted repeatedly with cold 0 45 *M* NaCl) accounted for about 10% of the total dermal collagen which is about equal to the daily increment in total collagen incident to growth Loss of weight for 2 days resulting from partial starvation caused a 50% decrease in neutral salt extractable collagen Orekhovitch has reported no decrease in citrate extractable collagen (procollagen) incident to starvation (27) Five to seven days of renewed continuous growth following a period of static body weight were required for the reappearance of the neutral salt extractable fraction (Fig 1) These experiments suggest that during active growth neutral salt extractable collagen is being actively synthesized when growth is interrupted synthesis ceases and the collagen formed previously has rapidly become insoluble in neutral salt solutions but is still soluble in acid solutions

Is there any structural or chemical difference between the collagen molecules extracted in weak acids and those extracted in cold neutral isotonic and hypertonic salt solutions? It is possible to dissolve neutral

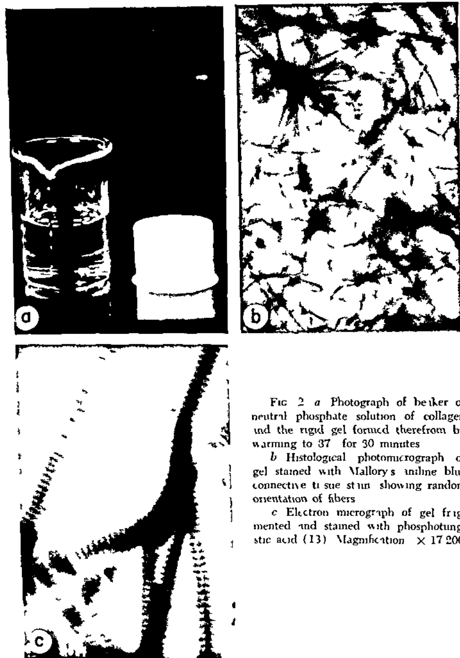


FIG 2 *a* Photograph of beaker of neutral phosphate solution of collagen and the rigid gel formed therefrom by warming to 37 for 30 minutes

b Histological photomicrograph of gel stained with Mallory's aniline blue connective tissue stain showing random orientation of fibers

c Electron micrograph of gel fragmented and stained with phosphotungstic acid (13) Magnification × 17 200

detachment by enzymatic action as in the fibrinogen fibrin system[1] It would appear that the difference between the collagens (extractable in acid alkaline or neutral media) are probably not in molecular structure or composition but rather in age of the molecules Thus Orekhovitch (28) Chia Mu and Tien Chin (2) and Bensusan and Hoyt (1) are only partly correct in suggesting that there is no difference between the collagen in neutral or acid solution The difference is biological rather than physical or chemical

There are however certain confusing aspects of the behavior of collagen in solution which suggest that either the small amounts of contaminants small differences in composition or different states of aggregation between the molecules have a significant effect on solubility As an example an acetic acid extract of ichthyocol from the carp swim bladder when dialyzed against citrate buffer at the same pH 3 5 will produce a precipitate of only part of the dissolved collagen dialysis of the supernatant fluid against water will produce another precipitate of fibrils having the same typical collagen axial periodic structure as did the first precipitate Jackson (22) has reported that hyaluronidase pretreatment of tendon greatly increases the amount of acetic acid soluble collagen but only slightly increases solubility in acid buffers Veis and Cohen (30) from extraction studies of collagen at elevated temperature conclude that there may be fractions of collagen differing in the type and degree of crosslinking between molecules and that some of the crosslinking may be covalent

A hint as to the possible mechanism for the changing solubility with time was found in an incidental but frequent observation by the writer that collagen in the form of wet fibers or dry lyophilized fluff from acid solution gradually become more insoluble with the passage of time even when kept cold (8) A systematic study was made of this phenomenon One of the interesting characteristics of the behavior of collagen dissolved in cold neutral salt solutions is its ability to precipitate as fibrils (characteristic of those of the native tissue) simply upon elevation of the temperature to 37°C (6 20) Bensusan (1) has recently discussed some of the kinetics of this phenomenon Nearly all the collagen is precipitated as a rigid opaque gel usually in less than 60 minutes (Figs 2a b and c) At first this was thought to be irreversible

[1] It is unlikely that there is any appreciable amount of a hydroxyproline containing particle with a low assymmetry ratio (such as a globular unit of collagen) in the neutral extracts since the intrinsic viscosity of the whole extract based on hydroxyproline esimate of collagen concentration is nearly identical with that of acid and neutral solutions obtained from purified collagen fibrils (10) Any significant amount of a globular" unit would lower this value

cooled and a third fraction could not be precipitated on warming (unless the ionic strength was elevated Fessler unpublished) Differences in structure or composition between these three fractions have not yet been detected The reversibility of the thermal precipitation of collagen was systematically investigated by the writer using, as starting material an acetic acid extract of calf skin dialyzed against cold neutral phosphate buffer a crude 0 45 M NaCl extract of skin of young rapidly growing guinea pigs and highly purified collagen isolated from a portion of crude extracts (11 12) The opacity of the gel formed on warming was directly related to the amount of collagen insolubilized hence it was possible to use opacity as measured in a Klett colorimeter as an index of the amount of precipitated collagen Cold solutions (phosphate NaCl buffer pH 7 6 ionic strength 0 4 and in other experiments 0 14) were warmed in a series of Klett tubes in a water bath at 37°C and the opacity measured at frequent intervals Opacity increased along a sigmoid curve reaching a nearly steady maximum after about 20–30 minutes Tubes were removed at intervals (after reaching peak opacity) of 10 minutes 30 minutes 1 hour 3 6 8 24 48 and 72 hours placed in an ice water bath and the opacity measured frequently Figures 3 and 4 illustrate the results of typical experiments At ionic strength 0 4 there occurred a rapid fall in opacity and liquifaction on cooling after 10 minutes incubation at peak opacity Increasing time of incubation at 37°C resulted in increasingly "irreversible"[2] precipitation The opacity data was supplemented by direct analysis accomplished by sedimenting and measuring the insoluble collagen remaining after 17–24 hours in the ice bath (All samples in any one experiment were kept in the ice bath for the same length of time) The relatively pure calf skin preparation the crude guinea pig extract, and the highly purified guinea pig collagen from the extract behaved qualitatively in the same manner The most pure collagen samples did show a greater degree of reversibility At physiological ionic strength irreversible precipitation developed much more rapidly than at higher salt concentration The same system was used to determine whether or not solubility changes in acid were also time dependent (Table I) Gels of collagen fibrils were formed in the same manner from the same three starting solutions Instead of cooling after varying intervals of incubation at 37°C the gels were dialyzed against 0 1 M citrate buffer pH 3 5 in the cold for 24 hours and the amount of sedimentable (insoluble) collagen remain

The term "irreversible" may not be quite accurate since it is not established yet that the insoluble phase is not in equilibrium with the dissolved protein The change here may conceivably be a diminished rate of solubilization at least for the relatively short periods of incubation

(6) but on occasion these gels were seen to lose opacity even redissolve upon storage in the refrigerator Fessler (4) had observed that one fraction of a neutral salt solution of collagen was irreversibly precipitated on warming a second fraction was freely reversible when

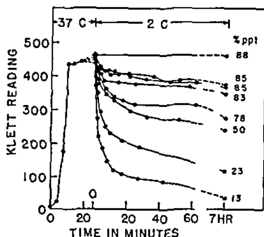


Fig 3 Effect of time of incubation at 37 C on rate and extent of solubilization of gel on cooling for 7 hours Calf skin collagen pH 7 4 Γ/2 = 0 45 NaCl Beginning with lowest curve in the 2 C phase (13% insoluble residue) times of incubation at 37 C were 20 minutes 1 3 6 24 48 and 72 hours (control not cooled) (12)

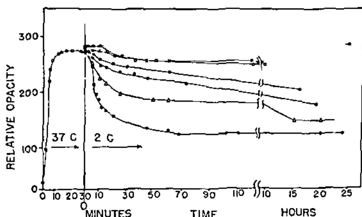


Fig 4 Effect of cooling to 2 C on the opacity of collagen gels incubated at 37 C for different periods of time Reading from the lowest curve upward ● 10 minutes △ 1 ◒ 3 ◓ 6 □ 24 x 48 and ○ ○ 56 hours (control not cooled) Starting solution contained about 0 2% calf skin collagen dissolved in cold 0 14 M sodium chloride buffered with phosphate to pH 7 6 (11)

dependent precipitation of tobacco mosaic virus protein (24) Once closer association takes place electrostatic bonds and Van der Waal forces become increasingly significant Because of the rigid structural organization of the collagen molecule there may very well be a "lock and key" type of aggregation resulting from the specific distribution of charged polar and nonpolar groups which would contribute greatly to the stability of the fibril This type of association could give rise to the typical axial periodic structure perhaps the more perfect it becomes the more insoluble the fibril Careful electron microscope studies correlated with the solubility behavior of fibrils formed *in vitro* might provide evidence to support this last speculation

These observations suggest a relatively simple explanation for the differences in extractability of collagen in weak acid and cold neutral salt solutions of different concentrations and the influence of age and growth rate Weak acids represent the strongest dispersing agents of this group probably due in part to their strengthening of the repulsive electrostatic forces between molecules by increasing the net positive charge Cold neutral salt solutions have a much weaker dispersing action which depends at least in part on the effect of electrolytes in weakening electrostatic bonds in aqueous media and also on the temperature effect on hydrogen bonding mentioned earlier The higher the salt concentration the greater will be the dispersing effect up to the point where "salting out" begins to occur This probably explains why physiological salt solutions extract much less collagen than do hypertonic solutions (6 10) Thus it would appear likely that cold physiological salt solutions would extract collagen which is most loosely or not at all aggregated hypertonic solutions would extract the above plus more tightly aggregated collagen perhaps in loose fibrillar form (or perhaps even in two or three dimensional networks) Dilute organic acids extract all of the above plus a considerable amount of collagen fairly well packed in the typical fibrils (a certain amount would be rendered unavailable by complexing with acid mucopolysaccharides at the low pH) This scheme is sketched in its general form in Fig 5 The biological significance here is that the collagen extracted with cold physiological salt solution is the most recently formed fraction that extracted with hypertonic neutral solutions includes somewhat older collagen and that removed with acid (procollagen) includes a large amount of even older collagen from the fibrils The insoluble collagen would represent the oldest protein which has become sufficiently crosslinked so as to resist solution in acid D S Jackson has discussed at this symposium data from his studies on C^{14} glycine incorporation in the collagen extracted under all these conditions and has been led from this other di

ing was determined. Complete solubilization occurred until sometime between 1 and 2 weeks of incubation at 37°C and neutral pH at which time most of the precipitated collagen had become insoluble (bacterial growth had been suppressed and possible effect of bacterial products had been ruled out analytically). Insolubilization of collagen in acid did occur more rapidly and completely in the crude connective extract than in the purified preparations suggesting an additive effect of interaction with noncollagenous constituents.

TABLE I
CHANGE IN SOLUBILITY OF COLLAGEN GEL IN COLD CITRATE (ACID PH) WITH TIME OF PREVIOUS INCUBATION AT NEUTRAL PH AT 37 C

Time of incubation at 37 C	Collagen gel insoluble in citrate (pH 4.5)			
	Calf skin (HAc extract)	Guinea pig skin (crude extract)		Guinea pig skin (purified extract)
	0.45 M NaCl	0.45 M NaCl	0.14 M NaCl	0.14 M NaCl
	(%)[b]	(%)[b]	(%)[b]	(%)
1 hr	0	63.7 ± 1.3	63.8 ± 4.8	0
6 hr	0	63.4 ± 1.2	64.2 ± 0.5	—
1 day	0	86.7 ± 1.1	67.5 ± 1.5	—
3 days	0	85.1 ± 0.5	78.1 ± 0.3	0
1 wk	0	89.3 ± 1.0	85.7 ± 0.7	45.0
2 wk	87.2 ± 0.2	95.0 ± 0.5	93.9 ± 0.9	56.0
3 wk	98.2 ± 0.1	—	—	60.0
4 wk	84.1 ± 0.9	95.6 ± 0.2	97.3 ± 0.9	—
8 wk	92.0 ± 1.0	—	—	—

Collagen isolated from crude NaCl extract

[b] Values in first three columns are the average of duplicates and the range between them.

These experiments suggest that at least one factor involved in the diminished solubility of collagen with age may be increasing numbers of secondary bonds between collagen molecules forming with time resulting from increasing perfection of fit between the molecules in the fibril or loose aggregate. This could result from Brownian movement permitting closer packing between identical molecules accompanied by the squeezing out of water. A reasonable explanation for solution of the aggregates in the cold might be the increased hydrogen bonding capacity of water at low temperature. In neutral solution at elevated temperature this factor is diminished and hydrogen bonding between protein molecules becomes more frequent resulting in aggregation. This explanation had been offered to account for the similar temperature

them "in solution" for varying periods of time? This question is of course related to the nature of the forces responsible for fibril formation The following experiments (7 13) represent a fairly simple approach again in the model system in an effort to provide some hints as to where one may look for answers To cold neutral salt solutions of collagen were added various substances in graded amounts and their influence on the rate and extent of thermal precipitation was determined (see Table II) Some of them were known hydrogen bond

TABLE II
AGENTS INFLUENCING RATE OF HEAT PRECIPITATION OF COLLAGEN[b]

Inhibitors		Accelerators	Ineffective agents
Arginine[a]	++++	SCN^-	Thiourea 0 2 M
Aspartic acid	++++	HCO^-	Glycine 0 2 M
Ascorbic acid	++++	I^-	β-Aminopropionitrile 0 ? M
Citrulline	+++	Br^-	α Glycoprotein 0 2%
Guanidine	+++	F^-	Gelatin 2 0 %
Urea	+++	Cl^-	
Glutamic acid	+++	Li	[d]Acid mucopolysaccharides
Glutamine	+++	Lysine	(with 2 exceptions)
Asparagine	+++	Ornithine	
Histamine	+++		
Creatine	+++		
Acetamide	+++		
Histidine	++		
Formamide	+		
Hydroxyproline	+		
Proline	+		

These agents were also tested at $\Gamma/2 = 0\ 14$ and 0 2 They had the same effect as at higher ionic strength but higher concentration of the agent was required

[b] The results shown are those obtained when tested at $\Gamma/2 = 0\ 4$ and phosphate pH = 7 6

Effectiveness of inhibitors relative to urea (+++) is indicated by the number of plus marks

[d] See Table III for details

Ornithine was mistakenly listed as an inhibitor in a previous publication (13) it acts as an accelerator as does lysine

formers such as urea and guanidine others were charged organic molecules such as amino acids and electrolytes such as the halogen salts Still others were simple metabolites such as histamine creatine and finally some large molecules such as acid mucopolysaccharides thought by some to be involved in fibrillogenesis Figure 6 illustrates the effect of graded, low concentrations of urea on the rate of heat precipitation in a solution of calf skin collagen in phosphate buffer pH 7 6 ionic

rection to essentially the same hypothesis It would appear likely that the loss of neutral salt extractable collagen with cessation of growth is due to cessation of collagen synthesis plus increasing insolubilization of the collagen formed at the end of the growth period for reasons discussed above Orekhovitch s observation of the diminution of acid extractable collagen in aging guinea pigs is probably a result of the lack of growth for a relatively long period of time The low specific activity of procollagen (acid extractable) in the isotope incorporation experiments is very likely due to the extraction of the neutral salt soluble fraction plus a large amount of unlabeled collagen from the fibrils formed before the administration of isotope

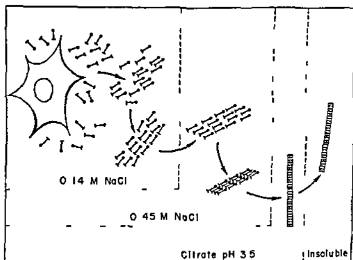


FIG 5 Schematic representation of an hypothesis formulated to help explain occurrence of the different extractable collagen fractions The rodlike units represent tropocollagen particles Cold physiological saline (0 14 M NaCl) extracts the most recently formed collagen molecules (and perhaps also that resulting from physiological degradation) which are completely dissociated or in the most loose association *hypertonic salt solutions extract the same material plus older collagen* in a more ordered state of aggregation acid citrate buffer extracts all of the above plus some of the older collagen in the typical fibrillar form The insoluble fibrils were of a sufficient age so that the degree of crosslinking has prevented solubilization

It seems likely that the model *in vitro* system of fibrogenesis which most closely approximates the *in vivo* formation of fibrils is the thermal precipitation from physiological salt solutions In the *in vitro* system the molecules were kept in solution by lowering the temperature Is there a physiological mechanism for regulating the rate of fibril formation from the newly formed complete molecules—some way of keeping

the sulfur analog of urea is ineffective as would be expected since it is a relatively weak hydrogen bond former Arginine (Fig 7) glutamic aspartic and ascorbic acids are even more potent inhibitors Lysine unexpectedly is a powerful accelerator of the thermal precipitation of collagen The common metabolites creatine and histamine are weaker inhibitors than is urea Hydroxyproline shows almost negligible inhibitory activity in the concentrations used a fact of some interest since the hydroxyl group of this amino acid is considered by Gustavson (15) to be important in the crosslinking by hydrogen bonds between collagen molecules Gelatin also has no effect

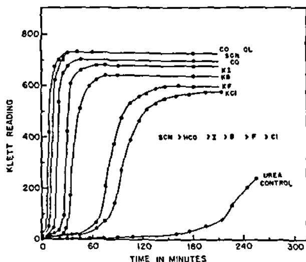


FIG 8 Comparative antagonistic effects of a group of anions on a urea inhibited solution of collagen Each preparation contained 0.25% collagen in phosphate pH 7 6 Γ/2 = 0 4 plus 0 24 M urea and 0 024 M of the test substances (13)

Curiously enough ions known to be powerful dispersing agents for proteins such as Li SCN I are in low concentrations strong precipitating agents for collagen KI for example will completely reverse the inhibitory effect of a tenfold greater concentration of urea The anions investigated seem to operate in the fashion of a reverse Hofmeister series (Fig 8) A variety of different hyaluronic acids chondroitin sulfates keratosulfate and heparitin sulfate from different tissues and obtained from different laboratories[3] were investigated in 0 2% and

[3] The author is indebted to Drs K. Meyer A. Balazs and R Jeanloz for generously providing these mucopolysaccharide preparations and to Dr Karl Schmid for the sample of α_1 serum acid glycoprotein

strength 0 4 As little as 0 01 *M* delays the process At physiological ionic strength it takes two or three times this concentration to achieve the same effect Urea 0 5 *M*, completely inhibits fibril formation however this is reversible as demonstrated by dialyzing out the urea Thiourea

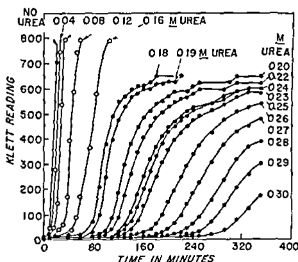


Fig 6 Effect of low concentrations of urea on heat gelation of 0 26% collagen solution in phosphate buffer pH 7 6 Γ/2 = 0 4 Two separate experiments are reported as open and solid circles The control behaved the same in both (13)

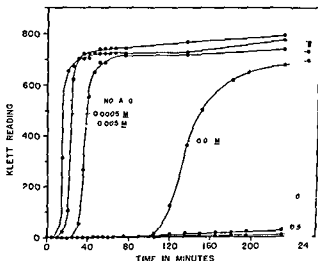


Fig 7 Effect of arginine in low concentrations on heat gelation of a 0 26% solution of collagen in phosphate pH 7 6 Γ/2 = 0 4 (13)

cules in solution as compared with the reverse situation prevailing when these molecules are degraded to gelatin Gelatin forms aggregates on cooling and disperses when warmed The difference here must reside in the rigid structural specificity of the collagen molecule which is destroyed upon gelatinization The unexpected behavior of lysine and ornithine for example as compared with that of similar basic amino acids such as arginine probably also depends on the structural specificity of the collagen molecule It would be very hard to predict the effect of any particular substance on fibrillogenesis at this time With regard to the exclusive formation of fibrils with the 640 A (native) axial periodicity (or structureless material) under the conditions of these experiments one may only state that this probably represents the most stable state of aggregation for the particular molecular structure of collagen We hope for detailed explanation eventually!

It would appear that the common acid mucopolysaccharides found in connective tissues do not obviously influence fibril formation at least under the conditions prescribed here (with two uncertain exceptions) However in view of the increasing variety of acid mucopolysaccharides found in the connective tissue a definite judgment on this point must be suspended until we have them all It should also be realized that the behavior of isolated and purified mucopolysaccharides in the *in vitro* system which lacks so many of the other normal constituents may not be a fair reflection of their behavior in the intact tissue It is quite possible that mucopolysaccharides play a significant role in the organization of the connective tissue but not necessarily in the process of collagen fibril formation They may be involved in the organization of the fibrils into tissue fabrics and may even be involved in determining the diameters of fibrils Meyer (25) has suggested that this might be the case with regard to the histologically visible fibers We should also not forget the relatively large amount of ground substance proteins of unknown nature which in amount in some instances far exceeds that of the mucopolysaccharides If physiological rate regulating factors for fibrillogenesis do exist perhaps the low molecular weight tissue components including common metabolites deserve closer scrutiny

Aside from the information bearing directly on the metabolism and development of collagen the *in vitro* model system discussed here (and others like it) may be usefully employed in elucidating some of the underlying principles of morphogenesis on the molecular level (7)

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TABLE III
MUCOPOLYSACCHARIDES TESTED FOR EFFECT ON THERMAL PRECIPITATION OF CALF SKIN COLLAGEN

Mucopolysaccharide	Tissue source	Obtained from	Effect
Hyaluronic Acid	Vitreous	A. Balazs	none
Hyaluronic Acid	Vitreous	A. Balazs	none
Hyaluronic Acid	Umb. cord	A. Balazs	none
Hyaluronic Acid	Umb. cord	A. Balazs	none
Hyaluronic Acid	Cocks comb	A. Balazs	Pptd. collagen in cold at Γ/2 = 0.4
Hyaluronic Acid	A Hem. Strep.	K. Meyer	none
Hyaluronic Acid	Umb. cord	K. Meyer	none
Chrondroitin SO_4 A	Cartilage	R. Jeanloz	Delayed ppt. at Γ/2 = 0.14
Chrondroitin-SO_4 A	Cartilage	K. Meyer	none
Chrondroitin-SO_4 B	Bov. lung	R. Jeanloz	none
Chrondroitin SO_4 B	Pigskin	K. Meyer	none
Chrondroitin-SO_4 B	Bov. lig. nuch.	K. Meyer	none
Chrondroitin-SO_4 C	Umb. cord	K. Meyer	none
Chrondroitin SO_4 C	Chrondosarcoma	K. Meyer	none
Chrondroitin-SO_4 C	Shark cartilage	K. Meyer	none
Kerato sulfate	Bov. cornea	K. Meyer	none
Keratosulfate	Hum. rib cart.	K. Meyer	none
Heparitin-SO_4	Bov. lung	K. Meyer	none
Heparatin SO_4	Liver gargoyle	K. Meyer	none

Conditions: pH = 7.6, Γ/2 = 0.14, 0.2, 0.4.

0.5% concentration at physiological and hypertonic ionic strength for effect on the rate or extent of heat precipitation (Table III). One hyaluronic acid sample from the cocks comb induced gelation and opacity in the cold within a few hours at Γ/2 = 0.4 but not at Γ/2 = 0.2. One sample of chondroitin sulfate B (from bovine lung Winterstein preparation obtained from R. Jeanloz) consistently delayed precipitation of collagen at Γ/2 = 0.14. Curiously the degree of inhibition and the characteristics of the opacity-time curves using this preparation were identical at 0.2 and 0.5% mucopolysaccharide concentrations. β-Aminopropionitrile (BAPN), the lathyrus factor which has been shown to increase the amount of neutral salt extractable collagen in rats (3) and chick embryos (24a) had no effect on heat precipitation.

These results suggest that both hydrogen bonds and electrostatic forces are responsible for fibril formation and that certain small molecules can profoundly influence the rate of precipitation apparently without irreversible alteration of the protein. This statement, it will be recognized, does not begin to approach the explanation for the negative temperature coefficient of precipitation shown by intact collagen mole-

ner in which the hydroxyproline groups can hydrogen bond In Model I the hydroxyproline residues are so situated stereochemically that the hydrogen bonds can be used *intra*molecularly i e between the three chains and in Model II the hydrogen bonds cannot be utilized *intra*molecularly but may be used *between* ad jacent macromolecules i e *inter*molecularly Although Model II appears to be pre ferred by many crystallographers recent physicochemical data obtained by Drs Nishihara and Doty at Harvard indicate that the hydroxyproline proline hydrogen bonds are used primarily *intra*molecularly which would fit the Rich Crick Model I

DR GROSS That is so

CHAIRMAN SMITH You cannot have the same one right on both counts

DR GROSS Yes that is the interesting possibility Within the fibrils you might very well have both forms

CHAIRMAN SMITH Except that the X ray diffraction pattern does not change does it?

DR GROSS No but this question of whether the hydroxyl groups are inside or outside is not completely settled The requirements for the model are less exacting if the hydroxyl groups of hydroxyproline are facing out

DR MEYER What holds the three chains together?

DR GLIMCHER Hydrogen bonds between other groups There is a hydrogen bond between the three chains Hydroxyproline is not the only thing that can hydrolyze

DR MEYER What about this enormous stability of the transformation of col lagen to the parent gelatin?

DR GLIMCHER No that is the point that is confusing you Parent gelatin does *not* consist of the three chain helical macromolecules randomly coiled When the three chains are intramolecularly hydrogen bonded they exist as stiff elongated rod like particles Parent gelatin results from the dissociation of these three chains so that they are completely unwound and separated in solution with a molecular weight approximately one third of collagen You really have to draw it on the board to see the possibilities since in *cold gelatin films* (i e not in solution but in solid state) some preparations of the three strands may reaggregate into the typical collagen three strand helix but not completely

DR GROSS The current hypothesis is three chains wound around each other the length from top to bottom being 2900 A When parent gelatin is made some how this thing is snapped in several places and the helix unwound

CHAIRMAN SMITH The various substances you are using make it appear as though the salt linkages are probably more important than hydrogen bonding

DR JACKSON Gustaffson has shown the salt linkages play quite a minor role in intramolecular linking

CHAIRMAN SMITH Yes but as far as internal molecular effects go salt linkages seem very important

DR GROSS Arginine probably operates by competing for the acid groups on the other molecules and aspartic acid would do the same for the basic groups But what about the effect of urea guanidine etc?

CHAIRMAN SMITH Arginine is a pretty weak hydrogen bonder with anything And you are certainly not going to get very much van der Waals effect

DR MOON Does this mean that once collagen is laid down and built into a large aggregate there is no further metabolic turnover?

DR GROSS I think metabolic turnover of collagen occurs only when remodeling is going on In bone for example as the animal ages there are changes in shape

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Discussion

Chairman Smith If the Rich Crick model is correct then all hydroxy groups of hydroxyproline are already hydrogen bonded so these cannot bind molecules together If the hydroxyproline hydroxy group binds individual molecules together then is the Rich Crick model wrong?

Dr Gross No There are two models proposed by Rich and Crick One has the hydroxyl groups of hydroxyproline outside and the other inside If the model with the hydroxyl groups facing out is right then interaction between molecules through the –OH groups of hydroxyproline could occur

Dr Glimcher There are two Rich Crick models differing mainly in the man

skin is broken in a Waring blender into the finest particles In the dark field microscope little chunks of jelly are seen but no fibrils Treatment with trypsin will then produce fibrils in abundance If an old man s skin is fragmented nothing is seen but a pure culture" of long clean straight fibrils I think in the young animal fibrils are separated into thin bundles As the ground substance is removed the little bundles come together mechanically and form big ones I do not think this is related to solubility changes They refer to the fibril and the molecules

DR ANGEVINE Would it be possible to look at this tissue and then extract and look at it again?

DR GROSS Yes we have done that

DR ANGEVINE And see which size thing is disappearing?

DR GROSS This sounds easy but there is a tremendous statistical headache here Thousands of fibrils must be measured with many preparations and sections and then maybe a statistical distribution of some significance may be had I did that once in a study of the effect of aging on fibril width In newborn rats the fibrils are very thin As the animal gets older more thick fibrils appear Why this is I do not know I personally think the ground substance is different at different ages with fewer fibril nuclei formed and larger fibrils produced This is just a wild hypothesis What we do know is that after NaCl extraction for example the fibrils appear perfectly normal in the electron microscope After citrate buffer extraction *a la* Orekhovitch the fibrils are completely destroyed Rarely can any be found with striations at all

DR WHITE In your diagram in which you showed fibroblasts discharging the little procollagens I assume there must be physiological factors influencing the orientation of these procollagens that is they do not come together in a random way? Do you know what these factors might be? Have you looked at skin of animals treated with growth hormone?

DR GROSS No but I can speculate I think mucopolysaccharides are laid down early in the formation of connective tissue as well as proteins of other kinds Maybe some are asymmetric But whatever asymmetric molecules are laid down of which the mucopolysaccharides may be a most important member they can be oriented by streaming by electrical forces by fibroblasts moving through them or putting out pseudopods etc The orientation of the matrix thus laid down beforehand may be an important factor in producing orientation of the collagen molecules perhaps even influencing the diameter of the fibrils as well as their direction

The various portions of the bone are replaced and remodeled I wonder whether some of Dr Boucek s data might not be explained on the basis of continual remodel ing When an object is put into a tissue and modifies its shape there is always an attempt to return to the original form as with the carrageenin granuloma

DR WHITE You said remodeling in terms of shape What about splitting and reconstituting? Do you mean insoluble collagen goes back to procollagen which then reforms aggregates in another way?

DR GROSS No The insoluble collagen is probably broken right down and re moved possibly by enzymatic activity of some sort about which we know nothing I wonder whether the molecules in mature fibrils can be dissociated physiologically to a salt soluble state

DR WHITE Most of the isotope data indicates collagen probably heads the list of body proteins that do not turn over

DR JACKSON This may be true but one should remember that only a limited number of connective tissues have been studied and of these only skin has been dealt with thoroughly Studies of skin bone liver and aorta do indicate a very slow turnover However there may be local difference For example in bone which is undergoing remodeling in one part collagen may be in the process of removal while synthesis is taking place nearby For the whole bone this may indicate a slow turnover

DR WHITE Have those experiments been done?

DR JACKSON Yes and differences can be shown between collagens from differ ent connective tissues More detailed studies on connective tissue other than skin are needed

DR GROSS Skin tendon and liver have been used

DR MEYER I believe some of the confusion here results from the fact that the behavior of collagen in solution *in vitro* has been used as a model for what is happening *in vivo* Older tissue becomes coarse and the more fine fibers tend to disappear Fibers are quite fine in bone The collagen bundle is highly organized and highly complex not an array of collagen molecules held together by hydrogen bonding or van der Waals forces etc One should see whether the submicroscopic bundles or the finest microscopic bundles first dissolve while the rest are un touched or react extremely slowly To make these analogies from the model experi ments *in vitro* to the tissue just does not go

DR JACKSON What I was discussing occurs at the molecular level To get a true turnover one has to show active replacement of a particular amino acid as representative of what is happening to the collagen molecule There may be a number of reasons why collagen fibers are resorbed or degraded and this is not strictly speaking turnover That was my point about the situation in bone

DR GROSS We are talking about three levels One is whole tissue one is bundle and the other fibril The fibril concerns us here We know nothing about the organization of fibrils into fibers We are trying to get at the mechanism whereby molecules form a cross striated fibril In this process I believe the inter molecular forces are primarily secondary linkages Perhaps there are a few ester linkages The organization of the fibers in the tissue is another phenomenon and maybe that is where the mucopolysaccharides and the ground substance play their role We do not know

DR MEYER But this is a process which takes place in aging

DR GROSS Yes but when you see big bundles forming the ground substance may be gradually removed as we know it is For example if a newborn baby s

The Macromolecular Aggregation State of Collagen and Biological Specificity in Calcification[1]

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I INTRODUCTION

Tissue mineralization or the deposition of inorganic crystals in an organic matrix is widespread in biology Examples of mineralized tissue can be found both in the plant and the animal kingdoms ranging from the most primitive to the most highly organized species Classic illustrations are the exoskeletons of certain marine molluscs (shells of clams oysters etc) and the endoskeletons (calcified cartilage and bone) of vertebrates The new structural properties which this deposition of crystals confers on these specialized tissues allows them to perform a number of different and varied mechanical functions as well as providing a storehouse of inorganic ions which may be used to help maintain the constancy of the ionic environment of the organism

While this process is normally restricted to specialized tissues such as bone cartilage and tooth alterations of the precise biochemical and biophysical control mechanisms may occur leading to mineralization of other tissues with serious consequences to the organism In the case of man these pathological calcifications occur in such tissues as skin tendons ligaments and the cardiovascular system It is this latter phenomenon which is of immediate interest to this group However it is obvious that in order to understand the pathogenesis of pathological calcification in the many different conditions in which it occurs an understanding of the factors involved in normal calcification is imperative I would there

[1] These studies were aided by research grants E 1469 (C1) from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health and A 2317 from the National Institute of Arthritis and Metabolic Diseases United States Public Health Service and 12A from the Orthopedic Research and Education Foundation Many of the major concepts involved have already been dealt with in a previous paper (Glimcher M J Hodge A J and Schmitt F O *Proc Natl Acad Sci U S* 43 860 1957) The detailed evidence and documentation for these concepts are currently in press

[2] Special Post Doctoral Research Fellow of the National Heart Institute National Institute of Health United States Public Health Service

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fore like to present a general theory of mineralization (with particular reference to the calcification of bone and cartilage) and provide a physical chemical framework within which it is possible not only to enumerate the factors responsible for the localization of the crystals to certain tissues but in addition indicate the circumstances under which it is possible for aberrant calcification to occur in terms of basic physical chemical parameters

To avoid any misunderstanding and confusion I would like to define certain terms The process of depositing a substantial amount of inorganic crystals in or on an organic matrix is defined as mineralization In this particular case since the crystals contain calcium the familiar term calcification is often used This is to be distinguished from ossification *which is bone formation and which includes the entire process* whereby certain cells lay down a specific matrix which is potentially calcifiable and whose tissue structure and organization is identifiable as bone tissue by ordinary histological methods

II The Composition and Molecular Structure of the Major Components of Bone

Analytically on a dry weight basis bone consists roughly of 65 to 70% of the inorganic crystals of the calcium phosphate salt apatite and 30 to 35% of organic matrix of which collagen makes up the major fraction (95 to 99%) (10) Dr Schmitt described in detail the molecular structure of collagen its macromolecular organization into fibrils and their further organization to form fibers fiber bundles etc so that we need not dwell on this particular point here The collagen in bone appears to be structurally similar to that in other tissues although it is probably highly cross linked[3] The other components in the organic matrix include a number of so far ill defined proteins as well as the acid mucopolysaccharides which in the case of adult compact ox bone *is chondroitin sulfate A (25) It is likely that in the tissue the chon* droitin sulfate is complexed with noncollagenous protein as a mucoprotein similar to cartilage (29 43) The exact anatomical location of these components at a macromolecular level is not certain and their state of aggregation and polymerization is not well known

Although it has been known for nearly two hundred years that bone contains calcium and phosphate it was not until 1926 that DeJong (9) demonstrated by X ray diffraction that the crystal structure was similar to the apatites more specifically hydroxyapatite $Ca_{10}(PO\ )_6OH$ the structure of which is illustrated in Fig 1 Although the exact nature of

[3] Author's unpublished observations 1957

the apatite in bone is still currently being debated for purposes of the present discussion we will refer to it simply as an apatite or hydroxyapatite

The highly ordered structure of the tissue at a microscopic level is best illustrated by examination of the compact bone of a long bone with which you are all familiar The basic unit is the *Osteon* or *Haversian* system These consist of lamellae or layers of bone concentrically arranged around a central canal which contains blood vessels These "cylinders" of bone are longitudinally oriented in the general direction of the long axis of the bone

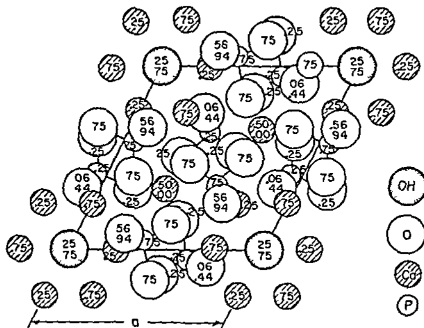
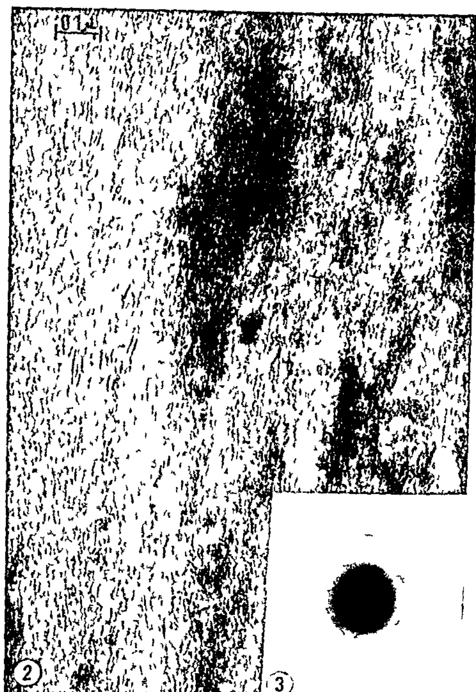


FIG 1 The atomic arrangement of the constituents of hydroxyapatite projected on the 001 plane The numbers refer to the fractional height in the unit cell of the atoms in the plane perpendicular to the paper (c axis) as reported by Posner (31) and Carlstrom (7)

Despite extensive investigation the arrangement of the collagen fibers and bundles in the lamellar system has not yet been completely elucidated (13 18 38 40 53) In general however the differences noted by many investigators appear to be merely ones of degree and undoubtedly represent variations of a basic scheme of organization These studies have revealed that the collagen fibers in a lamella are


0.1 μ
2
3

arranged in small bundles which encircle the canal in continuous spirals and which cross each other and result in a trellislike arrangement Although the general direction of the bundles in the same lamella is similar it varies from one lamella to the next In some Osteons the consecutive layers show fiber directions which are alternately circumferential and longitudinally disposed while in others the fiber directions are all oblique but at some angle to each other This change in the orientation of the fiber bundles in adjacent lamellae is the basis for the characteristic appearance of the Haversian system in cross section in polarized light

The inorganic crystals of apatite are deposited in this highly organized ordered matrix of collagen and ground substance The structural order evident in the arrangement of the macroscopic trabeculae of bone and in the microscopic Haversian systems is also evident at the next lower order of magnitude as well Starting with the early observation of Schmidt (40) with polarized microscopy many years ago it has been noted that both the collagen fibers (and presumably the fibrils) and the minute inorganic crystallites were cooriented Recent evidence from X ray diffraction electron microscopy and electron diffraction has shown that the crystallites are arranged with their long axes (crystallographic c axes) closely parallel to the fiber axis of the collagen fibrils (8 33 36) Figure 2 is an electron micrograph of a longitudinal section of compact bone The collagen fibrils are well oriented and the 640 A axial repeat of the fibril is accentuated in many areas by the specific location of the dense inorganic crystals The crystals are well oriented with their long axes parallel to the long axes of the collagen fibrils and intimately related to them Electron diffraction (Fig 3) confirms that it is the crystallographic c axis of the inorganic crystals which is parallel to the fiber axis of the collagen

Most workers have felt that the inorganic crystals of adult bone were in the ground substance between the collagen fibrils (18 33) although more recent work has indicated that some of the crystals might be within the fibrils (36) Interpretation of electron micrographs has been difficult however because of the dense packing of the collagen fibrils

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FIG 2 Electron micrograph of a longitudinal section of compact bone Note the accentuation of the characteristic axial repeat of the collagen fibrils in some areas by the small inorganic crystals whose long axes approximately parallel the collagen fibril axis Magnification × 100 000

FIG 3 Selected area electron diffraction of the specimen in Fig 2 showing characteristic pattern of apatite Arcing of the 002 and 004 reflections indicates that the crystallographic c axis of the crystal are oriented parallel to the collagen fibril axis and therefore correspond to the long axes of the crystals

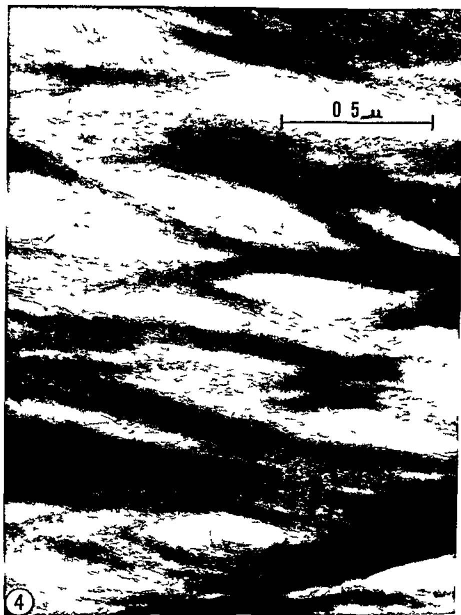


Fig 4 Electron micrograph of fish bone in a region of loosely packed fibrils Despite variations in individual fibril direction the long axes of the inorganic crystal are parallel to the individual collagen fibrils with which they are associated by virtue of their position within the fibrils Magnification × 75 000



Fig 5 Higher magnification of an area in Fig 4 The crystals appear to be rod shaped approximately 200–400 A long and 15–40 A wide Magnification × 225 000


0 1 μ
500 A
7
6

(Fig 2) From such electron micrographs it is not possible to say with certainty whether the crystals are within or on the surface of the collagen fibrils or just generally associated and oriented with them by virtue of the over all organization orientation and dense packing of the fibrils in the tissue We have recently resolved this issue by studying bone in which the collagen fibrils are sufficiently separated so that it is possible to see the relationship between individual fibrils and individual crystals by high resolution electron microscopy

Figures 4 5 6 and 7 are longitudinal and cross sections of such bone at various magnifications and clearly indicate that the crystals are primarily within the collagen fibrils and not in the intervening ground substance That this is not an initially random crystal precipitation between the fibrils later reorganized within the fibrils by recrystallization is evident from an examination of electron micrographs of embryonic bone (20) Figures 8 and 9 are electron micrographs of the initial stage of calcification in embryonic bone taken by Dr Sylvia Fitton Jackson of King s College London England These clearly show that the earliest crystals are regularly spaced and also deposited within the collagen fibrils

In view of the obvious association between the apatite crystals and the collagen fibrils in normally calcified tissues it seemed of interest to investigate the possibility that this intimate anatomical relationship was evidence that either the macromolecular structure of collagen or the macromolecular organization of collagen was responsible for the induction of calcification (15)

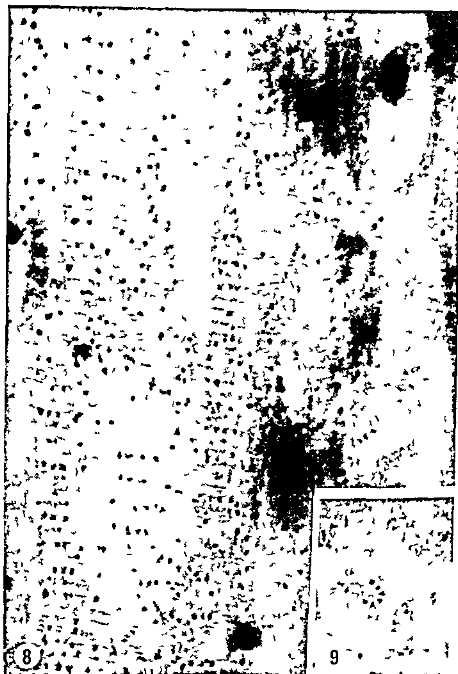
Other studies had also suggested a role for collagen (47) or collagen chondroitin sulphate complexes in calcification (44 45)

Because of the many biological complexities in tissue mineralization it is important that the physical chemical nature of the process be clearly defined In this case crystallization or the formation of inorganic crystals from solutions where none previously existed represents a phase change This physical change in state can be arbitrarily divided into crystal nucleation the process of forming the initial fragments of the new phase and crystal growth the subsequent growth of these fragments into clearly defined crystals In addition the phenomenon of recrystallization (the growth of large crystals at the expense of smaller ones) may also play a role even after the solid state has been achieved Failure to distinguish

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Fig 6 Higher magnification of cross section of fish bone The apatite crystals are obviously within the collagen fibrils Magnification × 165 000

Fig 7 Cross section of two fibrils in an area similar to Fig 6 The crystals have the appearance of rods viewed on end and appear to be hexagonally packed Magnification × 250 000



Figs 8 and 9 Electron micrographs of avian embryonic bone in the earliest stages of calcification Note the regular and periodic arrangement of the dense apatite crystals within the collagen fibrils Reproduced from original electron micrographs (20) courtesy of Dr Sylvia Fitton Jackson

these interrelated but separate phenomena and failure to differentiate the many regulatory processes controlling them from the underlying mechanisms can and has led to some confusion in the past Since the problem of induction of mineralization is primarily one of phase transition and to serve as a background for the experiments and hypotheses which follow a brief review of some thermodynamic and kinetic principles as they relate to phase changes will be presented

III Thermodynamics and Kinetics of Phase Transformations

Thermodynamically a system is said to be in a state of equilibrium with respect to certain changes in state when the sensible properties which describe that system do not change with time In the case of crystallization from solution the possible variation of interest is the formation of new phases

Although a system is either in equilibrium or is not (the case of the excluded middle is an explicit condition) many systems which meet the general conditions and criteria of equilibrium may vary widely in their relative stability to change in this case the formation of a new phase

It is the criteria and conditions of equilibrium as regards their stability with respect to the formation of new phases which is pertinent to the present problem Gibbs distinguished four kinds of equilibria based on this relative stability (14) These are (*1*) stable equilibrium (*2*) neutral equilibrium (*3*) unstable equilibrium and (*4*) metastable equilibrium They may be simply illustrated by mechanical models (Fig 10)

In the first case that of stable equilibrium consider a marble in a hemispherical bowl fitted with a cover (Fig 10a) Subject to the restraints that it cannot sink through the bowl or be removed from it a state of stable equilibrium exists with the marble at the bottom of the bowl for despite any possible displacement of the marble it will return to its original position In other words there exist no other states which result from either infinitesimal or finite displacements of the marble which are more probable (higher entropy) or for which the potential energy is lower The equilibrium of the marble is therefore stable The formation of new phases is not possible from solutions in stable equilibrium unless the entire system is changed (lifting the cover off and putting the ball elsewhere for example)

In the case of neutral equilibrium (Fig 10b) if the marble is rolled up along the sides of the tube or lifted from the bottom of the tube it will also tend to return to its position at the bottom of the trough However there do exist states as represented by positions of the marble lateral to its initial position which represent equally probable states (equal entropy) and states of equal potential energy Thus there will

be no spontaneous return of the marble to its original position if such horizontal displacements are made Solutions in such a state of equilibrium are also stable with regard *to the formation of new phases*

Unstable equilibrium may be illustrated by a marble at rest at the highest point on an inverted hemispherical bowl (Fig 10c) Even for infinitesimal as well as finite displacements the marble will assume a new more stable position This type of equilibrium is rarely attained *in practice and such solutions would hardly remain in such a precarious* state of equilibrium for very long periods It is important to remember however that while the marble is at rest in its position at the highest point on the bowl it is in true equilibrium albeit an unstable one

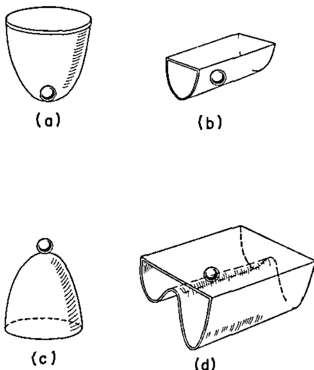


Fig 10 Schematic representation of the types of equilibrium based on their relative stability (a) stable equilibrium (b) neutral equilibrium (c) unstable equilibrium (d) metastable equilibrium

Of greatest interest to us is the state of metastable equilibrium represented schematically by a marble in the higher of two depressions of a continuous surface (Fig 10d) Despite all possible infinitesimal displacements the marble will return to its original position This is also true for certain finite displacements However there are some finite varia

tions possible represented by rolling the ball over the intervening hump which result in the marble assuming a new more probable position (higher entropy) and one of lower potential energy at the bottom of the lower depression It is obvious that the degree of metastability may vary (represented by the height of the intervening hump) and that at its outer limit the limit of essential instability (represented by the marble at the top of the intervening hump) the system becomes unstable Solutions in metastable equilibrium although capable of remaining stable indefinitely are capable of forming a new more stable phase under certain conditions

The next consideration is the problem of under what circumstances such metastable solutions are able to form these new more stable phases

The formation of the initial fragments of the new more stable phase from and in the presence of the original metastable phase is called nucleation Phenomenological theories of nucleation first developed for condensation (2 52) have subsequently been applied to a large number of phenomena including crystallization in which the change in state involves a phase transformation In the most general use such theories consider that local fluctuation of certain properties in initially homogeneous phases lead to the formation of clusters of molecules (or ions) of varying size structure and composition referred to as embryos The cluster of critical size structure and composition which is capable of further spontaneous growth and therefore of initiating the formation of a new phase is called a nucleus (Fig 11)

Such embryos are considered to arise by the stepwise addition of single molecules (or ions) i e a bimolecular mechanism and therefore the order of the reaction is equal to the number of molecules (or ions) in the nucleus

In most cases these "intermediate phases are considered to have the same composition and structure as the stable phase and differ from it only by their small size This permits a more simple derivation of many of the basic thermodynamic and kinetic relations The distribution of embryos of a given size is determined by Boltzmann s Law where the change in free energy [$\triangle F(r)$] refers to the work required to form a new phase of spherical clusters of radius r Thus the number of clusters of a given size n (r) is given by $n\ (r) = N \exp\ [-\Delta F(r)/kT]$ where $N =$ the total number of molecules constituting the system so long as the number of embryos remains relatively small Since the ratio of surface to volume is very much greater in these embryos and nuclei than in ordinary macroscopic particles their surface free energy constitutes an important part of the total energy required to form the new phase Considering only the interfacial free energy differences the work of forming these

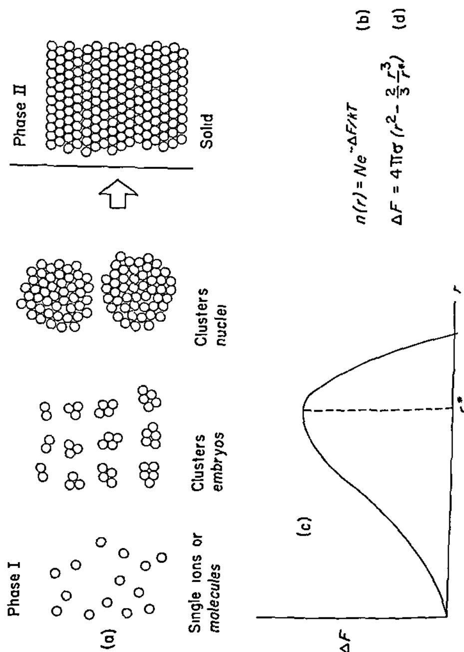


FIG 11 Diagrammatic illustration of crystallization from solution indicating the progressive growth of the embryos to the critical size of a nucleus (a) Equation (b) indicates how the distribution of clusters of a given size is related to their corresponding free energies The curve (c) shows the dependence in free energy change per cluster on cluster size and demonstrates the free energy barrier which the system must surmount for the formation of a new phase The curve correspond to equation (d) where σ = surface tension

embryos [$\Delta\Gamma(r)$] can be easily derived. When this increase in free energy per cluster is plotted as a function of the radius of the clusters r (Fig 11) it is obvious that the over all free energy of this system must pass through a maximum. This maximum corresponds to the critical size cluster (nucleus) of radius r^* which can grow spontaneously since the free energy decreases with further increase in size.

This free energy barrier is similar to the activation energy of ordinary chemical reactions and therefore permits one to derive expressions for the rate of nucleation. Such derivations have been made and although they contain a number of uncertainties are quite helpful in illustrating the major factors which influence nucleation rate. The general form of such equations is such that $J \sim \exp(-1/\ln S)$ where J = nucleation rate and S = supersaturation ratio i.e. the degree of metastability. It is obvious from such expressions and from experimental results that the supersaturation ratio dominates the rate of nucleation. For example in the case of water vapor condensing to liquid drops (50) the time that must elapse for the appearance of the first droplet at a supersaturation ratio of 4 is 0.1 of a second. Increasing the supersaturation ratio to 5 decreases the time to 10^{-13} seconds and decreasing the ratio to 3 increases the time to 10^{7} years!

In general two types of nucleation processes are described: homogeneous nucleation and heterogeneous nucleation. Homogeneous nucleation occurs in the bulk of the original phase in the absence of foreign inclusions and is due to violent local transient fluctuations which give rise to the clusters previously described, some of which reach the critical size, composition and structure of nuclei and are thus capable of further growth spontaneously.

A phase transition initiated by foreign inclusions extraneous to the system is called heterogeneous nucleation. The formation of such new phases occurs on the surface of such heterogeneities. An example of both types of nucleation may be illustrated by the phase transition of water to ice. Water may be undercooled to about −39° to −41° C if great care is made to eliminate all dusts and foreign particles (39). At −39° to −41° C the spontaneous formation of ice crystals is noted in the bulk of the water. Now if AgI crystals are added to the system before undercooling has begun the water need only be supercooled to about −4° to −6° C *before crystallization of ice begins* (39). In this case the crystallization of ice starts on the surface of the AgI crystals.

From many such observations Turnbull and his associates have developed a crystallographic theory of heterogeneous nucleation based on the similarity in atomic arrangement and lattice *spacings of the nucleation* catalyst and the crystals being nucleated (49). It predicts that the order of catalytic potency is proportional to the reciprocal of the disregistry between the catalyst and the forming crystals on low index

planes of similar atomic arrangement Thus AgI with a remarkably close fit between its lattice structure and that of ice is the most potent catalyst known for the nucleation of ice crystals Other factors such as the presence of surface defects and dislocations as well as interaction terms dependent on the nature of the chemical bond in the crystal etc must also be considered in the quantitative formulation of this theory

From a kinetic viewpoint heterogeneous nucleation may be considered a catalytic phenomenon the heterogeneity acting to increase the rate of nucleation The fact that in certain cases this increase is apparently discontinuous (nonfinite to finite) involves no contradiction concerning the original state of equilibrium since in such cases the initial rate (without catalyst) may either be so slow with respect to the length of the experiment that no change in state is assumed to be occurring (and theoretically therefore a system not in equilibrium but considered so for the purposes of the experiment) or the system may be in true *metastable equilibrium (and therefore independent of time) the heterogeneity* by acting as a discontinuous change in state initiates a phase change with a sensible and finite nucleation rate The fact that some heterogeneities will not act as catalysts at all is related to the nature of the discontinuity thus provided and its relation to any possible forces restraining change This is something only a detailed examination of the particular process involved will reveal

The phenomenological description of the manner in which phase changes occur clears up some misconceptions concerning the formation of apatite crystals from solution It has been assumed (24 27 48) that in the precipitation of apatite crystals the formation of brushite ($CaHPO_4 \cdot 2H_2O$) must occur first later to be hydrolyzed or otherwise converted to apatite [$Ca_{10}(PO_4)_6(OH)_2$] This is based on the incorrect premise that an 18 body collision is required for hydroxyapatite formation while only a 2 body collision is required for brushite formation

There are several conceptual errors here which need clarification In the first place when calcium and phosphate ions in solution aggregate to form a crystal the change in state which occurs is a physical change in state i e a change in the state of aggregation of the ions from a solution phase to a solid phase The empirical formulas of the solids in such cases [$CaHPO_4 \cdot 2H_2O$ or $Ca_{10}(PO_4)_6(OH)_2$] do not refer to a molecule or molecules of brushite (containing 2 ions and 2 water molecules) or apatite (containing 18 ions) but merely represent the ratios of all the constituent ions of the solid phase in terms of smallest whole numbers and/or in terms of the number of atoms in a unit cell The ions are part of a crystalline solid containing thousands or usually millions

of ions arranged in a definite spatial configuration characteristic of the particular crystal

Secondly even in chemical reactions where a physical change of state does not occur neither the reaction order nor the reaction mechanism can be deduced simply on the basis of the chemical formulas of the reactants or reaction products In nucleation of new crystalline phases nuclei probably also arise by the stepwise addition of single molecules atoms or ions (2 50 52) (bimolecular reaction mechanism) there being no obvious relation between the empirical formula of the solid and the size and composition of the nucleus or the order of the reaction

Inspection of the empirical formulas of various possible solids in no way permits one to predict the probability that the formation of one particular solid is more likely than another This of course will depend on the relative amount of work which is necessary for the formation of a cluster of a particular composition size shape and configuration under the specific conditions of the experiment i e pH temperature etc

Some simple examples will illustrate these points The formation of ice from water may be written H O (liquid) → H O (solid) Here of course it is obvious that we are not dealing with single molecules" of ice but with a solid containing many water molecules in a specific steric configuration It is also apparent that neither the size of the critical cluster nor the reactions kinetics can be deduced from the chemical formula or the equation which describes the change in state Physical chemical evidence (51) suggests that the nucleus which initiates the phase change is composed of about 80 to 100 water molecules It is highly unlikely that these nuclei arise by the simultaneous collision of 80 to 100 water molecules! The reaction is considered to be bimolecular in mechanism and therefore of the 80th to 100th order

The formation of sodium chloride crystals from solution is also illustrative and may be written as Na (ion aqueous) + Cl (ion aqueous) → NaCl (solid) Here again we have a physical change in state i e a change in the state of aggregation of the sodium and chloride ions from ions in solution to ion constituents of a crystal In this solid there are no NaCl molecules" but an array of Na and Cl ions with each sodium ion equally shared by six chloride ions and each chloride by six sodium ions The chemical formula of the solid (NaCl) obviously does not indicate that this change occurs simply as the result of a 2 body collision and is therefore a second order reaction

While there appears to be no doubt that under certain physical chemical conditions (low pH ~ 6 0–6 2) one can precipitate the calcium phosphate solid brushite and that this can in turn be hydrolyzed

or otherwise converted to apatite under the proper physical chemical conditions (raising the pH for example) it does not infer that when a calcium phosphate salt is precipitated the formation of brushite is more probable or more likely than apatite simply because its empirical formula contains fewer atoms and ions

IV The Mechanism of Crystal Induction

As Dr Schmitt has emphasized not only is the collagen macromolecule a crystalline protein but the collagen fibril by virtue of its being an ordered aggregation of such macromolecules and exhibiting such a high degree of structural regularity both by electron microscopy and low angle X ray diffraction may also be considered crystalline The obvious implication is that this highly ordered fibrous protein can act as a catalytic heterogeneity for the nucleation of apatite crystals In order for such a mechanism to be operative however the solutions from which the crystals are formed must be in metastable equilibrium In assessing the physical chemical conditions as they exist *in vivo* it is obviously important to establish the state of equilibrium of the fluids immediately surrounding the fibrils in relation to the formation of this new phase (apatite crystals) This poses a difficult problem Although it has been demonstrated that the bulk of the extracellular interstitial fluid is metastable with respect to the formation of apatite crystals[4] the local values immediately surrounding the collagen fibrils in any particular tissue might be markedly different due to active cellular controlled compositional changes or active or passive transport and diffusion phenomena *Such changes however are concerned with the regulation* and control of the process and not the mechanism of initiating the phase transformation The immediate objective is establishing whether collagen can induce the formation of apatite crystals from solutions which are

[4] The ion product $a_{Ca^{++}} \times a_{HPO_4^{--}}$ in the serum of many vertebrates is considerably higher than the same product in solutions of equivalent ionic strength pH temperature etc where equilibrium has been approached either through precipitations or dissolution of apatite crystals (47) *Since this product appeared to be* critical in determining whether or not precipitation of a solid phase occurred it was concluded that serum and interstitial fluid was supersaturated with respect to the bone mineral *While this thermodynamic data is not conclusive direct experimental* evidence supports this supposition Inorganic solutions with ion products $a_{Ca^{++}} \times a_{HPO_4^{--}}$ similar to serum were stable for indefinitely long periods of time but showed rapid separation of *more* solid when exposed to bone mineral (26) or to synthetic apatite crystals (Glimcher unpublished data) It would therefore appear that the unaltered extracellular interstitial fluid is metastable with respect to the formation of apatite crystals

metastable and to determine the nature of this specificity (if any) and its mechanisms The experiments which were conducted at M I T in collaboration with Dr Alan Hodge and Professor F O Schmitt were designed to answer these questions

Dr Schmitt has described how the collagen of many connective tissues can be dissolved in a number of weak acids and neutral buffers yielding viscous solutions of the macromolecules and how these can subsequently be reaggregated and reconstituted into fibrils with the typical and characteristic axial repeat and intraperiod fine structure of native fibrils By appropriate treatment of the connective tissue prior to dissolving the collagen and by several recrystallizations the collagen fibrils can be prepared relatively pure with only minute traces of ground substance constituents

The first experiments were carried out by exposing such reconstituted 640 A axial repeat collagen fibrils to solutions shown previously to be metastable with respect to the formation of apatite crystals Because of the technical difficulties involved and because the thermodynamic properties of calcium phosphate solutions and their relation to the formation of solid phases is not well enough characterized it has not yet been possible to procure quantitative data on nucleation rates crystal growth rates free energy of nucleation etc The more qualitative methods used were designed to detect the ability of the test materials to induce the formation of apatite crystals and depended mainly on the identification of the formed crystals They did not distinguish between nucleation rate crystal growth recrystallization etc

Following exposure to these metastable solutions the occurrence of nucleation and crystal growth was determined by a number of different methods Changes in the calcium and phosphate concentrations of the metastable solutions were followed as a function of time and compared with control solutions The collagen matrix was also analyzed for calcium and phosphate content both before and after exposure to the metastable solutions and was subjected to X ray diffraction which identified the nature of the inorganic material crystallographically when it was present Electron microscopy was used to visualize the crystals when present and to show the relationship between the inorganic crystals and the collagen fibrils In addition the crystals could be further identified by selective area electron diffraction

The results of these experiments demonstrated that native type 640 A axial repeat reconstituted collagen fibrils prepared from normally un calcified tissues such as rat tail tendon calf skin guinea pig skin fish swim bladder etc were able to nucleate apatite crystals from metastable

calcium phosphate solutions Figure 12 is an X ray diffraction pattern of such a calcified collagen preparation showing the typical diffraction rings of apatite

Ironically we were not able to prepare reconstituted collagens from bone presumably because of its highly cross linked nature However bone could be decalcified under a variety of conditions which main tained the 640 A structure of many of the collagen fibrils This prepara tion was also able to induce crystallization of apatite Although it is very difficult to make quantitative comparisons it appeared that many of the decalcified bones were not grossly as potent in this respect as the recon stituted collagens presumably due to changes in the structure of many fibrils and to alteration of certain important functional groups during decalcification (19 46)

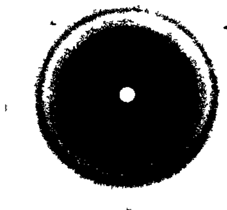


FIG 12 X ray diffraction pattern of *in vitro* calcified collagen Note the lack of crystal orientation (evidenced by complete rings) and the broadness of the diffraction lines due primarily to small crystal size

Since in nucleation phenomena a number of discontinuities are able to induce phase changes the next problem was to determine whether or not this property was specific for collagen and if so where the specificity lie Paramyosin fibrils another well organized and ordered fibrous protein structure showing a regular periodic pattern by electron microscopy and low angle X ray diffraction was obtained from the adductor muscles of clams and used in similar experiments under identical physico chemical conditions They were not able to induce this phase change

As you will recall from the preceding chapters a number of other fibrillar forms can be reconstituted *in vitro* from the same solution of

collagen macromolecules These include fibrils with 220 A axial periods structureless fibrils with no discernible band pattern fibrous long spacing (FLS) and segment long spacing (SLS) (Fig 7 of Dr Schmitt s chapter) As was clearly pointed out these are different ordered aggregation states of the same macromolecules and reflect differences in the packing and intermolecular geometry of the fibrils These differences are of course also reflected in differences in the kinds of amino acid side chains which interact and differences in their stereochemical relations to one another Since it was also possible to isolate the collagen macromolecules and linear polymers of collagen macromolecules (protofibrils) both in solution and in the solid state we were thus provided with a most unique system with which to determine the steric nature of the specificity Figure 13 is a schematic representation of the basis for these experiments Using SLS as a typical example with which to compare the native type fibril it is apparent that within any region of the fibril although the same macromolecules and therefore the same amino acids are present the steric relations between amino acid side chain groups is different

The experiments were carried out as previously described by exposing the macromolecules protofibrils and the various aggregation states to metastable Ca P solutions The results may be briefly stated Neither the macromolecules linear aggregation of macromolecules or any of the fibril forms other than those with the native type 640 A axial repeat were able to initiate this phase change under identical physical chemical conditions Since it was possible to pass reversibly from one fibril form to another and to demonstrate nucleating ability only while in the native 640 A form it was evident that in producing the other aggregation forms the macromolecules were in no way denatured

These experimental findings indicated that the process of induction of crystallization was a heterogeneous nucleation of apatite crystals from metastable Ca P solutions by the collagen fibrils and was dependent not on the macromolecules per se or in single linear adlineations of macromolecules but in groups of macromolecules and protofibrils polymerized laterally and longitudinally in a highly specific fashion characteristic of native collagen Thus it would appear that the particular type of aggregation and packing of macromolecules characteristic of native type fibrils creates highly specific steric relationships within the fibril which serve as centers for nucleation

Additional confirmation of the necessity of a specific juxtaposition of certain reactive groups was obtained by subjecting aliquots of reconstituted native type collagen fibrils and demineralized bone to a number of physical and chemical agents which altered or destroyed the inter period fine structure of the collagen fibrils without altering the reactive

side chains and demonstrating that the ability to induce crystallization had been lost These included the effect of heat acid and alkali In the case of heat no change was noted until the thermal shrinkage temperature was reached Thermal shrinkage of collagen results in a phase change as regards its state of aggregation similar to gelatinization This

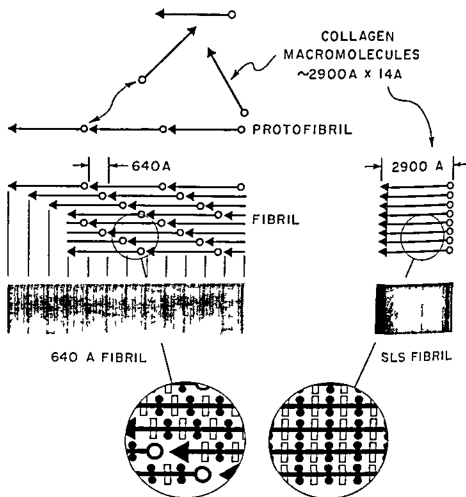


FIG 13 The steric differences between the reactive side chain groups of adjacent macromolecules in the native and SLS forms of collagen diagrammatically portrayed

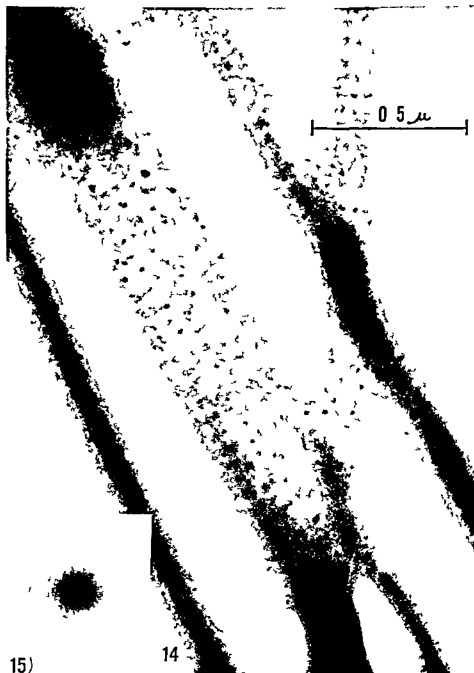
disrupts both the molecular structure and the macromolecular aggregation state of collagen In the case of acid treated demineralized bone or alkaline treated reconstituted fibrils although collagen fibrils undergo a good deal of swelling and distortion leading to loss of the typical 640 A

repeat by low angle diffraction and electron microscopy the macro molecular structure remains intact In both cases the amino acid side chains are not chemically altered but only their steric relations changed

With reference to the phenomenon of nucleation three distinctly different aspects of the relation between collagen and apatite must be considered The first is the stereochemical relationship between the reactive side chain groups which constitute a nucleation center The second is the demonstration that there are preferred nucleation centers and the determination of their location with respect to the known elec tron microscopic intraperiod fine structure of the collagen fibrils The third is the nature of the chemical groups in the nucleation centers and the nature of the intermolecular forces between these groups and the mineral ions

An undue emphasis seems to have been placed on the absolute value of 640 A characteristic of native type fibrils and its relation to nucleation and other calcification phenomena It should be apparent however that there is nothing specific about this absolute value since it is merely a visible manifestation of the particular aggregation state of the collagen macromolecules and acts as a "finger print" for the recognition of the native type fibrils Its importance lies in the fact that when the macro molecules are arranged in a specific three dimensional array so that this characteristic electron density distribution occurs along the fibril certain regions are created within the fibril whose reactivity and steric relations permit them to act as sites of heterogeneous nucleation It is this stereo chemistry between reactive side chain groups within the nucleating regions which is directly related to the mechanism of nucleation and this has no relation to the absolute value of 640 A

In attempting to correlate the molecular structure of collagen and the property of nucleation therefore one must clearly distinguish between the structural characteristics of the macromolecules and the higher order configurations due to the specific arrangement of the macromolecules in the fibril Unfortunately unlike the case of nucleation of inorganic crys tals by other inorganic crystals a direct correlation cannot be made be tween the wide angle diffraction spacings of collagen and the known lattice spacings of apatite as has been attempted by one group of work ers (28) The wide angle diffraction pattern of collagen cannot be treated as arising from simple Bragg planes as in inorganic crystals but must be interpreted on the basis of the theory of helical diffrac tors (6) Except for the 10 12 A equatorial spacings indicating the distance between the centers of adjacent macromolecules the wide angle reflections are characteristic of the triple chain helical coiled structure of the macromolecule and give no information as to the


0 5 μ
14
15)

interatomic distances and configurations of the side chain groups in the fibril which information is directly related to the *nucleation* phenomena It should be clearly understood that not only do the 640 A type fibrils give the characteristic wide angle X ray diffraction pattern but also the macromolecules themselves and all other fibrillar forms as well as cold gelatin films To date neither the exact sequence or stereochemistry of the amino acids in the collagen macromolecule are well enough known to permit determination of this specific configuration although some general statements may be surmised from other data to be presented

As regards the second point the demonstration of specific nucleation centers and their location we have attempted to elucidate this aspect by several different methods Direct visualization by electron microscopy and measurement of changes in electron density either in electron micrographs or from low angle X ray diffraction patterns have been tried[5] Interpretations based on electron micrograph density changes and low angle X ray diffraction density changes are fraught with technical and theoretical difficulties and no definite statement is yet possible from these data

Electron micrographs taken during the course of time sequence studies of *in vitro* calcification (Figs 14 and 15) and during the earliest stages of *in vivo* calcification of embryonic bone (14) (Figs 8 and 9) provide direct evidence that there are specific regions in the fibrils which act as nucleation centers These show that during the earliest stages of calcification *in vitro* and *in vivo* small dense particles varying in size from 30 A to 150 A are deposited regularly spaced along the fibrils Selected area electron diffraction of these particles in both cases revealed that they were crystals of apatite (20) (Fig 15)

Since the smallest crystals visualized in electron micrographs represent the summation of nucleation and crystal growth interpretations based on such visual observations must be viewed with some caution

FIG 14 Unstained unshadowed preparation of an early stage of *in vitro* calcification of collagen fibrils Note the regular and periodic distribution of the crystals along the collagen fibril axis corresponding to the intraperiod fine structure of the fibrils and occurring primarily once per axial period Magnification × 80 000

FIG 15 Selected area electron diffraction pattern of such a preparation showing the characteristic apatite reflections There is no evidence of preferred crystal orientation even though the diffraction pattern was obtained from an area where the fibrils were well oriented

[5] Many of the low angle X ray diffraction studies were carried out in cooperation with Dr Aron Posner American Dental Association Research Division National Bureau of Standards

It is entirely possible that regions most favorable for crystal growth may be quite different from those where nucleation occurs There may also be different areas of nucleation but differing in their degree of catalytic potency

Mention should also be made that it is not an entire band" or inter band in the collagen fibrils which represents a nucleating center but only regions within such a transverse section of the fibril This can be easily deduced if one considers that it takes a certain number of repeating groups from adjacent macromolecules in just the right configuration to make a nucleation center and that statistically there will only be a finite number of such groups within any one transverse section

While it has not been possible to identify the exact location of the nucleation centers with respect to the intraperiod fine structure of the collagen fibrils observations thus far indicate that these centers do correspond to certain of the so called "band" (electron dense) regions This would correlate well with related chemical and low angle diffraction studies (1) which have shown that the "band" regions are the most reactive sites for many electron stains tanning agents etc and that these areas probably contain many of the amino acids with long reactive polar side chain groups In this respect it is interesting to note the recent demonstration of the direct correlation between the degree of mineralization and the reactivity of the epsilon amino groups of lysine in bone and tooth during demineralization (46) since lysine is considered to be a major constituent of the band regions (21)

While these experiments have indicated the nature of the specificity in the heterogeneous nucleation of apatite crystals by native type collagen fibrils and emphasized the importance in this particular case of steric factors they do not provide information concerning the manner in which these steric factors operate or the nature and importance of the chemical interaction between collagen and the mineral ions

Certain general principles may be stated however Thus while it is obvious that some sort of chemical interaction must occur between certain reactive groups in the collagen fibrils and the appropriate ions in order for nucleation to occur it is imperative that the nature of the forces involved be such that the bound ions are still capable of interacting with the other constituent ions of the crystal lattice For if either calcium or phosphate ions were very strongly bound by the collagen fibrils collagen would act as a demineralizer similar to chelating agents unless a second mechanism were invoked which released the ions after they were bound

There are several distinct ways in which the steric factors could operate to induce crystallization The simplest case would be by facilitating the local concentrations of calcium and/or phosphate ions without requir

ing that the ions themselves be arranged in any particular steric fashion This local increase in ion concentration would lead to crystallization by exceeding the metastable limit Another possibility is that the precise array of groups necessary for nucleation imparts a specificity for the selective adsorption or binding of the calcium or phosphate ions either randomly or in specific stereochemical fashion and that they then interact with the other constituent ions of the lattice to produce the first fragments of the new phase A third method would be the most specific of all and would statistically require the fewest number of atoms In this case the precise array of reactive groups would sterically closely approximate certain low index planes of apatite in a fashion similar to that proposed by Turnbull *et al* (39 49) and discussed in an earlier section Thus calcium phosphate (and hydroxyl ions) either singly or in combination and dependent upon which atomic plane of apatite was involved would be lightly" bound in such geometric configuration that they constituted a reactive nucleus

Of course one must consider the possibility that certain clusters of ions are bound by the reactive groups in the collagen structure This is really a semantic issue however since the binding of such clusters which presumably have the configuration of the stable solid phase would require similar steric considerations

Experiments in which nucleation failed to occur after native type reconstituted fibrils and demineralized bone were alternately exposed many times to solutions containing either calcium or phosphate ions in concentrations equal to or several times greater than those of metastable solutions in which nucleation readily occurred confirm the supposition that the intermolecular forces between collagen and the inorganic ions must be just strong enough to affect their interaction energies without firmly binding them to the collagen structure

V The Localization Regulation and Inhibition of Crystallization

The phenomena of localization regulation and inhibition of the physical chemical mechanism which initiates crystallization both in normally mineralized and unmineralized tissues are different but closely related problems

On the basis of the hypothesis and experimental data thus far presented it would appear that all collagenous matrices are inherently capable of nucleating apatite crystals from metastable solutions Since collagen is the major fibrous protein of all the connective tissues (skin tendon ligaments etc) the questions arise why don t all these tissues normally calcify and why do they calcify in certain pathological conditions? Also even under normal circumstances apatite crystals are de

posited in tissues which do not contain collagen fibrils (enamel) and in abnormal states in other noncollagenous tissues as well

While it is impossible to answer all of these questions definitively at the present time the controlling factors and the circumstances under which they may be operative can be developed within the framework of the physical chemical concepts of solution metastability and heterogeneous nucleation sites In a conference such as this I also feel that it is important to attempt *to correlate the basic research studies with the particular clinical problem at hand* even if to do so requires a bit (and maybe more) of speculation However only by such relatively uninhibited expressions of ideas can a useful interchange be accomplished between scientists and physicians working in a number of different fields

1 *Localization of the Crystals to Specialized Tissues*

We do not mean to suggest that collagen is the only organic crystal capable of nucleating apatite crystals This is quite unlikely as in all nucleation processes many materials can act as nucleation catalysts with varying degrees of potency

In the case of tooth enamel the apatite crystals are closely associated with another structural and "crystalline protein a eukeratin (3 42) and it is likely that a similar mechanism for crystal induction exists here

2 *Regulation and Inhibition*

Since the native collagen fibrils of most connective tissues do *not* calcify under normal conditions one of several general situations or a combination of them must exist Either the collagen in those tissues not normally mineralized is different from that of bone or calcified cartilage or the degree of metastability of the extracellular fluids immediately surrounding and within the collagen fibrils in the various tissues is different or other local phenomena increase the catalytic potency of the collagen fibrils in the normally mineralized tissues

Since reconstituted native type collagen fibrils from a wide variety of *tissues normally not calcified were able to initiate crystal formation in vitro* in our experiments failure to mineralize if attributed to the collagenous component would involve subtle structural differences between native and reconstituted fibrils as yet unresolved by the physical and chemical methods thus far employed It is possible however that during the extraction and reconstitution of the fibrils parts of the collagen macromolecules are lost which normally *in vivo* inhibit calcification There is no data available with which to evaluate the feasibility of this suggestion

With respect to the second possibility that differences in *the* degree

of solution metastability account for the specific localization and regulation of calcification two different points of view are possible If one assumes that the degree of metastability of the unaltered interstitial fluid is sufficiently great so that collagen can induce perceptible rates of nucleation some mechanism must be operative in the normally unmineralized tissues which prevents crystallization If the degree of metastability of the unaltered interstitial fluids is not sufficiently high an increase in the degree of metastability or an increase in the catalytic potency of the collagen fibril is necessary for crystallization even in the normally mineralized areas and a minimal protective mechanism is required in the normally uncalcified regions

These variations in the degree of metastability could result either (a) from cellular controlled compositional changes in the interstitial fluids or (b) from the presence of other substances in the tissue which either actively or passively controlled diffusion and specific ion transport and transfer or competed with the mineral ions for active sites in the collagen fibrils

Cellular controlled compositional changes of the interstitial fluids include variations in the calcium and/or phosphate concentrations calcium to phosphate ratios pH ionic strength ion complexes etc These could be mediated directly by the cells or by substances secreted by the cells such as enzymes For example the demonstration that phosphorylative glycogenolysis can produce a local increase in phosphate concentration in epiphyseal cartilage (33) illustrates the role of such a device in the regulation of calcification in normally mineralized tissues either by merely making more phosphate available or by participating in another enzymatic cycle (67) which then actively transfers phosphate ions to specific groups in the collagen fibril

As to the other method of regulating the degree of metastability (particularly in normally unmineralized tissues) by controlling diffusion ion transport etc the possible role of the ground substance and certain of its components has also been investigated

Although much research has been done on the ground substance of connective tissues its exact composition state of aggregation and anatomical distribution are still not clear Prominent among its components are the various acid mucopolysaccharides These are thought to exist in the tissues complexed with noncollagenous proteins (29 43) In the past most investigators have postulated that of the ground substance components the chondroitin sulfates specifically either alone or in combination with collagen have played a role in the initiation of calcification (12 37 44 45) That it is not solely the amount of the acid mucopolysaccharides which determines whether calcification is initiated

is obvious from the fact that hyaline cartilage one of the richest sources of this material is normally uncalcified while bone which does calcify contains extremely small amounts of these substances Differences in the kinds of mucopolysaccharides present is also not a plausible explanation since the various acid mucopolysaccharides found in adult bone or in the growing ends of bone (bone epiphyseal cartilage etc) are also present in other tissues (25)

It is instructive to review the findings as to the nature of the ground substance in cartilage since a transition occurs from the normally un calcified hyaline portion to the normally calcified epiphyseal region over a relatively short anatomical distance

When tissue sections of hyaline cartilage epiphyseal cartilage and bone are stained with metachromatic dyes or by the Hotchkiss procedure definite differences are noted (24) The staining characteristics of hyaline cartilage gradually change as the epiphyseal cartilage is ap proached This change is also evident in bone which is being actively deposited or resorbed Since metachromatic dyes react with negatively charged high molecular weight compounds these staining characteristics have been linked primarily to the sulfated mucopolysaccharides in such connective tissues The change in metachromasia and in the staining properties of these tissues with the Hotchkiss procedure has been in terpreted as evidence for the depolymerization of the anionic mucopoly saccharides as the zone of calcification is approached and reached (12) Although there are obviously a number of other possible reasons for this change in staining properties the important point for this discussion is that there is some alteration either in the amount state of aggregation or reactivity of charged groups etc in the ground substance and that this accompanies calcification Analytical data (23) which demonstrated a very marked loss of organic sulfate during cartilage calcification and the formation of bone matrix indicate that it is the depolymerization and subsequent removal of these compounds which are related to calcification

In attempting to assign a specific role to the ground substance in calcification it is helpful to examine it from a physical chemical point of view In this case two properties stand out which I believe are im portant in the process of calcification One of these is the fact that its state of aggregation is probably that of a gel in which the chondroitin sulfates exist complexed with a protein moiety as a mucoprotein (29 43) Such a physical state of aggregation might well limit the diffusion of interstitial fluids and ions to the collagen fibrils

The anionic groups of this mucoprotein are free and reactive (41) which accounts for the second property of importance the large cation

binding capacity of the ground substance Under normal circumstances therefore the mucoproteins may help inhibit calcification by limiting the available mineral ions both by diffusion and selective cation (calcium) binding It is also possible but less likely [since collagen and the chondroitin sulfates for example do not react with collagen above pH 4 0 (11)] that the reactive groups of the mucopolysaccharide portion of the mucoprotein compete with the mineral ions for position in the collagen fibrils but it is possible that the noncollagenous protein portion of the mucoprotein may interfere with the process by just such a mechanism

Depolymerization and removal of these compounds would eliminate this diffusion barrier and decrease the cation binding capacity of the ground substance allowing the mineral ions to react with the collagen The cation binding capacity of the remaining depolymerized mucoprotein components would also most likely be decreased since it has been shown that metallic cations are more strongly bound to high molecular weight acids and bases than to their monomeric compounds (16)

Several other experimental observations indicate the validity of this hypothesis Hyaline cartilage rich in the acid mucopolysaccharide protein complex fails to mineralize *in vitro* but selectively removes Ca from calcifying solutions (4) This property has been shown to be due to the sulfate groups of the chondroitin sulfates

The tanning and dyeing of skins which depends on the interactions of certain dyes etc with specific groups in the collagen fibrils is markedly facilitated by extraction of the ground substance and much more so by the prior depolymerization of the chondroitin sulfates by testicular hyaluronidase (5)

The hypothesis is further supported by a series of *in vitro* experiments which we conducted in which native collagen rich tissues such as rat tail tendon calf skin guinea pig skin etc failed to mineralize under physical chemical conditions identical with those used in testing reconstituted native type fibrils from these same tissues but when treated so as to extract many of the components of the ground substance (including the chondroitin sulfates) either directly or by enzymatic depolymerization these same native tissues readily mineralized

In vivo the rapid depolymerization of the anionic mucopolysaccharide protein complexes and the subsequent decrease in their cation binding properties might well lead to a local release of free cations including calcium with a resultant increase in calcium concentration locally thus further increasing the degree of metastability

Figure 16 is a schematic representation of many of the experiments

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safely controlled It seems likely that a compromise is utilized by maintaining the degree of metastability in unaltered extracellular fluid normally just below the point where collagen is easily effective

On the one hand the rate of nucleation in bone and cartilage could then be increased and controlled at a perceptible rate by very small increases in the local concentration and/or transfer and transport of mineral ions by enzymatic mechanisms for example such as that described earlier for phosphate ions the prevention of pathological calcification could be assured by further decreasing the degree of metastability by utilizing the properties of the ground substance previously enumerated

There is every reason to expect that like other functions of such vital importance to the organism mineralization is under the control of many such factors delicately balanced to provide maximum efficiency and protection Together they help provide the biological and cellular controlled regulations of the physical chemical mechanism which initiates crystallization

3 *Pathological Calcification*

From what has been discussed thus far it is obvious that a change in a number of different variables alone or in combination could result in the calcification of tissues normally unmineralized With reference to Figure 13d the most direct way would be by simply increasing the degree of metastability so that the spontaneous precipitation point (limit of essential instability) is exceeded No catalytic surface is needed at all (rolling the ball over the hump) On the other hand it may only be necessary to increase the degree of metastability without exceeding the limit of essential instability With this increase in metastability the protective devices in the tissue may be overwhelmed and collagen (or for that matter other less potent catalytic surfaces) would induce crystallization In the same fashion the relative degree of metastability within or on the surface of the collagen fibrils might be increased by permitting the normally metastable extracellular fluids access to them This could be accomplished if the components in the tissues which normally regulate this were either removed or their state of aggregation changed Obviously there are any number of such combinations possible Time is not available to discuss all the many kinds of pathological calcification but I would like to mention some data which is related to pathological calcification of the aorta

Several studies have shown that there is a marked decrease in the hexosamines hexuronic acid and organic sulfate of the hyaline plaques of arteriosclerotic arteries (30 47a) one of the most common sites of

thus far described It illustrates both the specificity of the collagen macromolecular aggregation state in initiating calcification and the possible role of the ground substance as a factor in inhibiting it

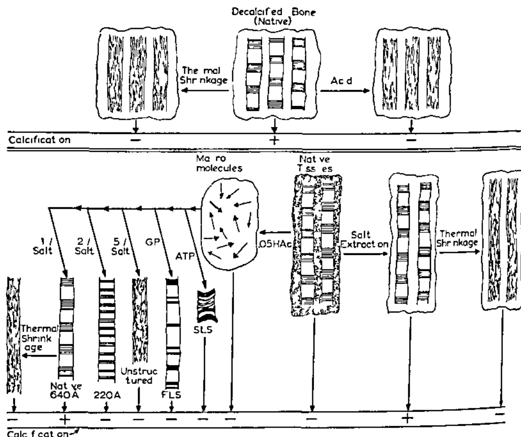


Fig 16 Composite and diagrammatic illustration of the experiments demonstrating the specificity of the macromolecular aggregation state of native type collagen fibrils in calcification and the possible role of the ground substance (heavy stippled regions surrounding fibrils in native tissues) in inhibiting and controlling it Enzymatic treatment of the native tissue is not shown since it is not yet certain whether depolymerization is itself effective or whether depolymerization and subsequent removal from the tissue is necessary

Since nucleation rate is so markedly dependent on the degree of metastability (p 111) the organism has the dual problem of keeping the metastability of the extra cellular fluids at a high enough level for collagen to induce a *perceptible rate in normally mineralized tissues* and at the same time not so high that aberrant calcification cannot be

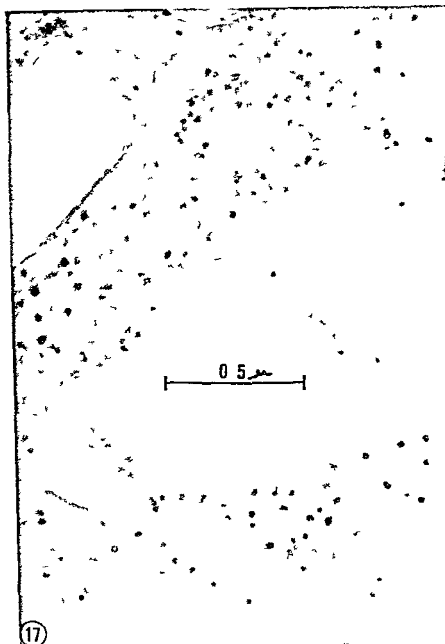


Fig 17 Unstained unshadowed preparation of *in vitro* calcified collagen at an early stage Taken out of focus so that the crystal reflections (white spots) can be visualized It is apparent that these are haphazardly arranged indicating lack of preferred orientation of individual crystals with the fibrils with which they are associated Magnification × 70 000

pathological calcification This is interpreted as due to a depolymerization and subsequent removal of the sulfated mucopolysaccharides These analytical studies have been correlated with changes in the staining properties of the arteriosclerotic plaques (32) From the preceding discussion the implications are apparent

VI Crystal Orientation

The experiments which I have described were carried out in a model system and although we are fully aware of its limitations it has enabled us to characterize the process in fairly well defined physical chemical terms as well as demonstrating the rather remarkable steric specificity involved In addition however it has also been possible to distinguish several closely allied phenomena and to gain an insight into their mechanisms One of these is crystal collagen co orientation

As was mentioned earlier the long dimension of the inorganic crystals in bone closely parallels the collagen fiber axis However in calcifying cartilage the crystals are randomly oriented (34)

Since the process of orientation was felt to be a true oriented overgrowth directed by certain crystallographic planes in the collagen fibrils (epitaxy) (7 54) and directly related to the process of mineral phase induction it was suggested that possibly different mechanisms were involved in the initiation of calcification in these two closely related tissues (28) *This was also based on electron microscopic evidence* that in the zone of provisional calcification in cartilage the size appearance and organization of the collagen fibrils was markedly different from that in bone (34 35) More specifically the collagen fibrils in this calcifying zone of cartilage are quite thin (50 to 250 A) and do not show any visible interperiod fine structure The fibrils are in addition widely separated and randomly oriented Correlations of this type however depend both on an intimate knowledge of the mechanism of crystal orientation its relation to the induction of crystallization and on information concerning the macromolecular organization of the apparently structureless collagen fibrils

Electronmicrographs taken during time sequence studies of *in vitro* calcified reconstituted collagen have shown a striking similarity in the appearance of the inorganic crystals to those of embryonic bone during the initial stages of calcification (Figs 8 9 14 and 15) In both cases the crystals appeared "dot like" with none of the axes elongated in any direction Selected area electron diffraction of both embryonic bone and early *in vitro calcified collagens showed no preferred crystal orientation* It was impossible to obtain single crystal patterns from the *in vitro* calcified specimens although in many instances individual diffraction spots rather than complete rings were obtained from small local areas with

relatively few crystals and in regions where there were several well oriented fibrils

In order to obtain more information about the relationship of individual crystals to the collagen fibrils recourse was had to another experimental device The objective aperture of the electron microscope was removed and the specimen photographed slightly out of focus The resultant crystal reflections seen as white dots in Fig 17 are from a definite set of crystal planes confirmed by measuring the distance from the crystals to the reflections One can easily see the haphazard patterns of these reflections clearly indicating that the individual crystals are not oriented with their crystal axis parallel to the fibril axis but are randomly oriented This indicates that the initial crystals have not been grown in an epitactic fashion and that their subsequent orientation must be due to other factors As further experimental results unfolded it became apparent that the reasons for preferred crystal orientation were twofold the size and habit of the inorganic crystals and the location of the crystals within the fibrils

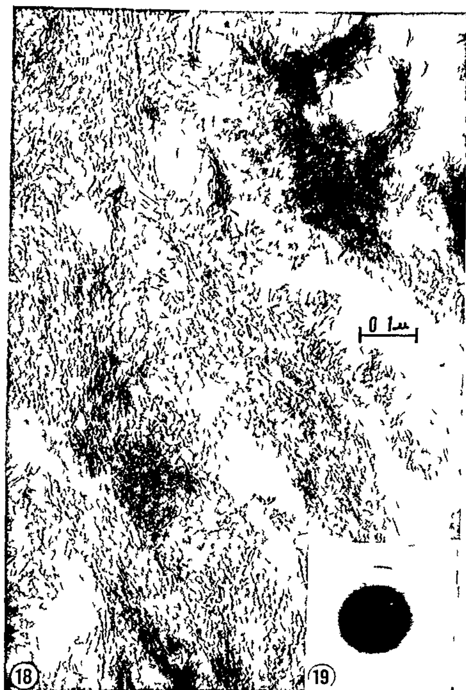
The data may be summarized as follows In both calcifying cartilage embryonic bone and *in vitro* calcified collagens crystallization is initiated in direct relation to the collagen fibrils During the first stage of mineralization in embryonic bone reconstituted collagens and calcifying cartilage the inorganic crystals are not oriented with respect to the collagen fibril axis In both embryonic bone and reconstituted collagens the crystals are strikingly similar "dot like" with none of the crystallographic axes elongated

Further crystal growth results in asymmetric crystals with one of their axes elongated In bone this occurs primarily within the fibrils and is associated with orientation of the crystals That the orientation of the crystals is not a function of the over all compactness or organization of the collagen fibrils in the tissue but is dependent on the location of the crystals within the fibrils is apparent from observations on embryonic bone (Figs 18 and 19) and certain adult bone (Figs 4 and 5) In the former despite the general randomness of the fibrils the crystals are oriented with respect to individual fibrils and in the latter although the

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FIG 18 Electron micrograph of a section of the metatarsal rudiment of embryonic chick bone (16 days) Despite the general randomness of the collagen fibrils the crystals are oriented in local regions corresponding to the individual collagen fibril directions The size and shape of the crystals is similar to adult bone Magnification × 140 000

FIG 19 Selected area electron diffraction of specimen shown in Fig 18 The preferred orientation of the crystals is evident by the arcing of the 002 and 004 reflections


0 1μ
18
19

Although the mechanisms by which mineralization is initiated and regulated in the organism are undoubtedly far more complex than those in the relatively simple model system described the process of heterogeneous nucleation of inorganic crystals by highly specific regions in the organic matrix due to the stereochemical array of certain reactive groups is probably a fundamental one not only in calcification but in biological mineralization in general

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collagen fibrils are clearly separated and somewhat randomly directed the inorganic crystals are likewise well oriented with their long axis parallel to the collagen fibril axis with which they are associated In calcifying cartilage however because of the very small diameter of the fibrils crystal growth is propagated primarily between the widely spaced fibrils and therefore these crystals in no way capable of being oriented by the fibrils are randomly dispersed The conclusions are therefore reached that (1) The basis for the orientations of the inorganic crystals is the asymmetric growth of the crystals within the collagen fibrils and therefore between tightly packed longitudinally oriented chains of macromolecules (protofibrils) which necessarily results in the crystals being similarly oriented (2) The process of crystal orientation therefore is unrelated to the mechanism of induction of crystallization (3) Since crystal orientation is a function of the size and shape of the crystals and their position within the fibrils and not part of the crystal induction mechanism the lack of preferred orientation in no way suggests that the mechanism of crystal formation (heterogeneous nucleation by the collagen fibrils) is different in cartilage than in bone

The other point concerning calcifying cartilage that is the lack of the usual interperiod fine structure of the collagen fibrils appears to be due to the very small size of the fibrils and possibly also to the large amount of ground substance surrounding them The question of collagen fibril size is also important from the standpoint of the number of collagen macromolecules that must be polymerized laterally in order to constitute a nucleus With these factors in mind reconstituted collagen fibrils were prepared which were approximately 50 A to 100 A in diameter and which did not show any cross striations when strained with phosphotungstic acid (PTA) Well oriented fibrils of this material however demonstrated the characteristic 640 A low angle X ray diffraction pattern *indicating that their macromolecular organization was similar* to the larger fibrils Preparations of these fibrils were able to initiate crystallization of apatite *in vitro* from metastable solutions in experiments similar to those already described

When one now examines the data concerning calcification of cartilage the theory of heterogeneous nucleation of apatite crystals by collagen fibrils is equally as convincing as in bone particularly compact bone where the collagen fibrils are so closely packed that there is so to speak very little space for the crystals to form except within the fibrils In cartilage on the other hand the fibrils are relatively widely spaced and randomly oriented with large amounts of intervening interfibrillar ground substance available for crystallization Despite this calcification does not begin randomly but is initiated in direct relation to the very thin fibrils and is then propagated throughout the intervening ground substance

has thus far not been possible to do a quantitative study of nucleation since it would involve measuring nucleation rates and distinguishing nucleation crystal growth etc This is a difficult problem

I would like to re emphasize that we used collagens from rat skin rat tail tendon guinea pig skin calf skin and the fish swim bladder and decalcified bone from four species and all of these were able to produce this change provided the long range fibrillar structure (640 A axial repeat) was intact Thus despite the fact that a wide range of analytically different collagens were used structurally they were similar as far as this particular property (apatite nucleation) is concerned

Dr Bolcek In the sponge implant in rats Dr Howell in our laboratory has examined by microincineration the location of calcium deposition It was interesting that calcium was precipitated at the interface between the sponge and collagen fibers with little seen in the other collagen of the section

Dr Gross Looking back at that story of Solomons and Irving is there a possibility that the reason why you might not get calcification in areas that normally do not calcify is that acidic substances might bind onto these lysin groups thereby eliminating the source of nucleation?

Dr Glimcher Although it is possible that the anionic groups of the chondroitin sulfates for example might effectively react with the epsilon amino groups of lysine I think it is more likely that the chondroitin sulfate portion of the mucoprotein complexes combine with the divalent calcium ions This is more likely since the available evidence indicates that there is no complexing of collagen and chondroitin sulfate at the pH of body fluid However it is possible that the active group in the collagen such as the epsilon amino groups of lysine and hydroxylysine might be bound by the *protein portion* of the mucoproteins thereby making them unavailable to the mineral ions

Dr Lansing Is it not true generally that the only place where one finds calcification or mineralization of white fibers in connective tissue is in cartilage or in bone? In the absence of necrosis which one finds occasionally in tendon or in other sites the white connective tissue does not calcify?

Dr Glimcher I think that is correct The relation of tissue necrosis to calcification I think has been best stated by Dr Hass of Chicago I believe he feels that while grossly it would appear that calcification occurs in necrotic areas actually it occurs in areas around the necrosis first and then as the tissue undergoes necrosis only the mineral is left But you apparently have to have an intact protein or organic matrix to initiate calcification

Dr Lansing Having flirted with microincineration for about twenty years where I find white fibrous connective tissue I do not find mineralization or calcification even in the arteries concerning us here It is not the white fibrous connective tissue that calcifies in fact it stands out in microincineration by the absence of calcium salts It is only when you find bony plates histologically demonstrable bone

Dr Glimcher I tried to bring out that *bone formation and calcification are* different entities

Dr Lansing All right mineralization

Dr Glimcher But bone is another story Ossification is the laying down of a definite histological structure and calcification is the mineralization of a matrix When you talk about white connective tissue this refers to a different hierarchy level than I was talking about You see once the seed is started you get propagation of calcification throughout at that point you cannot tell the relationships between the mineral and the matrix very well

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Discussion

Dr Boucek Did you observe any difference in the reactivity of collagens obtained from different aged rat tails?

Dr Glimcher We didn t run any studies directly relating nucleation capabilities of collagens and the age of the animals We did use very young animals and very old animals and the collagen that was reconstituted seemed to act just as well It

alternate exposure of the fibrils to solutions containing either calcium or phosphate at concentrations equal to and even several times greater than that of metastable solutions (where both were present) and from which crystallization readily occurred

Most investigators have felt that the initial step in calcification involved an anionic group which combined with the calcium A closer inspection particularly in light of recent data casts some doubt on the primary role of calcium binding in crystal induction

In the first place it is the concentration and active transport of phosphate ions which is presumably regulated by the enzymes and by their increase control the local degree of metastability Secondly the apatites are phosphate salts and the structural characteristics of the apatite lattice are primarily due to the phosphate groups not the calcium atoms which can be replaced by a number of cations without changing the major features of the crystal structure and symmetry Since the phosphate groups are the "backbone" of the lattice their role in the formation of the initial crystal structure would appear to be equally as important if not more important than the calcium ions The findings of Solomons *et al* previously referred to which showed a direct correlation of the available epsilon amino groups of lysine and the degree of mineralization of bone and tooth during demineralization also is very suggestive and may indicate that the primary collagen mineral ion interaction is between the epsilon amino groups of lysine and the phosphate ions There are obviously a number of other possibilities and experiments now under way in which specific groups are being blocked singly and in combination should provide the necessary data for the interpretation of the actual molecular mechanism of the nucleation process

Dr White But if you do preliminary single ion dialysis do you change the rate of subsequent nucleation of that collagen against which you have dialyzed?

Dr Glimcher We have never measured rate of nucleation I would suspect that you could but it would be difficult

Dr Moon I want to emphasize that calcification of arteries as observed by light microscopy begins in the internal elastic membrane and then spreads in either direction If one looks at arteries with that in mind for example the superior or inferior thyroid arteries which show early calcification and also in experimental animals under certain conditions calcium seems to be laid down rather massively in those areas before it spreads out elsewhere

Dr Wexler Our group has been producing experimental arteriosclerosis by inducing a hypercortical state As you look down the aorta the interesting thing is the great variety of lesions One conspicuous change is the amount of calcification There is a metastatic and dystrophic variety of calcification and even cartilaginous metaplasia which is quite unusual and bone formation *This occurs only in the* female There is a sharp sex dichotomy In our experiments to date this does not occur at all in the male

Let us assume that the matrix of the female and male aorta are essentially the same How would you explain this result?

Dr Glimcher I can t—I would just leave it as an observation!

Chairman Smith Maybe the regulatory processes *are enzymatic* as was suggested 25 or more years ago by Robison and that with one system we can regulate phosphate concentration You can use the phosphatases I think this is a hypothesis that still stands This is no quarrel with the nucleation hypothesis You still have to have nuclei to grow crystals Simultaneously the regulatory process can be enzymatic

DR LANSING Nevertheless the hypertrophied intima is very rich in collagen and reticulum

DR GLIMCHER Have you seen calcification outside the collagen fibrils?

DR LANSING No I have not seen recognizable calcium in or around the white fibrous connective tissue

DR GLIMCHER At what level in the intima? Where are the calcium crystals occurring in relation to the structure of the intima?

DR LANSING It is largely in the media not in the intima at all and when you find it in the intima it is in highly circumscribed areas which have undergone true bony change

DR GLIMCHER When they have bone that is a different story

DR LANSING But that is the only time you find calcium in the intima

DR GLIMCHER In other words in the intima you find only bone is that it? It is bone or merely calcification?

DR LANSING The loose connective tissue the intima does not calcify That is all I am trying to say

DR GLIMCHER But if you have only true bone formation then it has nothing to do with what we are discussing now We can conclude that the intima does not calcify it ossifies which is another thing It is a process of cell differentiation

DR LANSING Exactly

DR GLIMCHER Now in the media where is this calcification?

DR LANSING In the elastic tissue

DR GLIMCHER Right in the elastic fibers?

DR LANSING Yes

DR GLIMCHER Is there any collagen around?

DR LANSING Of course

DR GLIMCHER Do you know that it doesn t start in the collagen fibers?

DR LANSING Insofar as one can recognize by the existing methods of crude histochemistry and microincineration it is

DR GLIMCHER Microincineration certainly will not tell you where it is in relation to a fibril will it? I don t want to imply that calcification can not occur in elastin I have said that calcification can occur in any matrix if you increase the degree of metastability sufficiently It can occur in any organic matrix which is capable of acting as a seed But I would suspect if you were to look at the very first stages of nucleation of apatite in the arteries you would probably find it in very close relationship to the collagen fibrils

DR MEYER I think this is a matter of magnitude It has to be compared to your pictures of the electron microscope You would only see the nucleation if a lot of calcium is deposited The nucleation may start throughout even in the younger years and the problem is what limits the size of the crystal of the number of nucleation centers so you do not calcify throughout? What starts nucleation? The calcium ion or the phosphate? There must be some strong binding site

DR GLIMCHER Aside from the fact that a cluster or complex of calcium phosphate might very well be the initial moiety bound I think I should re-emphasize one important point *The type of bond or intermolecular force between the mineral ions and the collagen must necessarily be relatively weak* otherwise if either the calcium or phosphate ion were strongly bound collagen would act as a demineralizer similar to the chelate compounds This has been substantiated by our so-called "single ion dialysis experiments where we got no crystal formation despite multiple

simply because you found an increase in alkaline phosphatase in tuberculous lymph nodes is very little ground for extrapolating this to mean that calcification here or elsewhere is at all related to alkaline phosphatase In addition to say that osteoblasts affect bone formation by a specific process is really not saying anything at all about its mechanism! I don t wish to go into the matter further since you have not had a chance to present your data in full but I would like to state that I agree with the Neumans in their recent book when they comment that the ghost of Robison s theory (alkaline phosphatase) has finally been laid to rest!

CHAIRMAN SMITH Many ghosts come back

DR GLIMCHER Yes this ghost may come back to haunt me!

in vivo We do not have any enzymes that regulate calcium concentration but we do know a whole group that can regulate phosphate concentration

DR GLIMCHER There is no question and I think I tried to bring it out that these other phenomena which may be regulatory play an important role in calcification but they do not constitute the mechanism of getting ions out of solution and into a crystal lattice! As regards the specific role of alkaline phosphatase as first proposed by Robison about 25 or 26 years ago I believe there is no evidence that such a mechanism is operative Most workers in the field have pretty much discarded that theory and feel that its role if any is tied in either with the formation of the organic matrix or as a dephosphorylating synthesizing and/or phosphorylating agent as suggested by Gutman and Yu

DR ASTRUP I would disagree very definitely because we have been able to revive the theory of Robison At places where there is tissue necrosis particularly in connective tissue we have observed a release of formation of alkaline phosphatase even if the cells do not normally contain alkaline phosphatase The cells produce alkaline phosphatase when they are dying forming the basis for an unspecific calcifying process This can be demonstrated on tissue cultures of normal chicken heart fibroblasts which are connective tissue cells with no alkaline phosphatase in their normal cytoplasm (T Astrup and E Henrichsen *Exptl Cell Research* 6 151 1954 E Henrichsen *ibid* 11 115 403 1956) In this manner the degenerative calcification in tissue necrosis can be explained e g in tuberculous lymph nodes (E Henrichsen *Exptl Cell Research* 11 511 1956) Osteoblasts effect bone formation by a specific process while the calcification of cartilage is an effect of alkaline phosphatase produced by cell degeneration (E Henrichsen *Acta Orthopaed Scand* 27 173 1958)

Sometimes in tissue cultures it is seen that alkaline phosphatase will be deposited on the collagenous fibrils The alkaline phosphatase deposited by cell degeneration will regulate the local deposition of calcium phosphate which again forms the crystalline nuclei for further deposition of calcium phosphate by crystal growth The process of calcification can be divided into 3 or 4 definite stages

DR GLIMCHER Are you suggesting that alkaline phosphatase is the *primary* mechanism or a regulatory phenomenon?

DR ASTRUP A primary mechanism for producing crystals of calcium phosphate serving as nuclei for the deposition from a supersaturated solution and which regulates the position

DR GLIMCHER I might have let that pass if you considered it one of the regulatory mechanisms but to consider it the primary mechanism in this day and age is preposterous In the first place simple precipitation by exceeding a so-called solubility product would not account for the precise specificity of location within the collagen fibrils and in such a perfectly ordered fashion Secondly Gutman and Yu who have worked on this enzyme problem a number of years failed to find a substrate for alkaline phosphatase in epiphyseal cartilage Thirdly even if one postulates that in some mysterious unknown fashion alkaline phosphate increases the phosphate concentration it would never reach the level of spontaneous precipitation since it has been shown not only by our own studies with collagen but by a host of others using rachitic bone and epiphyseal cartilage as well as collagen where *no* alkaline phosphatase was present that mineralization occurs (by heterogeneous nucleation) *long* before such an ion product is reached

To comment on the experimental results which you mentioned I would say that

simply because you found an increase in alkaline phosphatase in tuberculous lymph nodes is very little ground for extrapolating this to mean that calcification here or elsewhere is at all related to alkaline phosphatase In addition to say that "osteoblasts affect bone formation by a specific process is really not saying anything at all about its mechanism! I don t wish to go into the matter further since you have not had a chance to present your data in full but I would like to state that I agree with the Neumans in their recent book when they comment that the ghost of Robison s theory (alkaline phosphatase) has finally been laid to rest!

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Some Ways by which ACTH and Cortisol Influence Functions of Connective Tissue

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The investigations reported here may not at first glance appear to have any relationship to the problem of the development of arterio sclerosis however the blood vessels are formed by cells which either are or are closely related to those of all other connective tissues It is true that morphological resemblances of cells does not imply that they are necessarily functionally the same However with the exception of smooth muscle (which much evidence indicates is closely related to connective tissue cells) the cellular and acellular constituents of vascu lar tissue are the same as those found in loose and dense connective tissue The cells and their products may differ in some ways but for the present it seems warranted to assume that at least certain principles of cellular behavior may be similar throughout the mesenchyme derived tissues

Because the pathological lesions in arteriosclerotic vessels contain much cholesterol and there appears to be a correlation between abnor mal amounts of this substance in the blood and the incidence of these lesions attention has long been focused on cholesterol metabolism However there are other alterations both in arrangement and composi tion of arteriosclerotic lesions which may also be important e g arterio sclerotic placques at early stages contain reticular fibers and muco polysaccharides (1) Later vascular alterations exhibit fibrosis and cal cification which is identical to that found elsewhere in the connective tissues of the organism Cartilage and bone formation may occur nor mally in the aging human aorta These processes including cholesterol deposition are common in nonvascular lesions in aging and patho logically altered connective tissues everywhere but assume particular importance in vascular tissue due to their capacities to limit adequate organic function

The data presented here from our laboratory is not as much con cerned with arteriosclerosis as such but with how certain steroid hor mones may influence certain functions of connective tissue cells

The cells of the connective tissue with which we have been most concerned are the fibroblasts histiocytes (reticuloendothelial cells) and mast cells In some instances it has been possible to compare some of the functions of these cells as they are modified by cortisol and other hormones

It has been demonstrated that cortisol and closely related steroids modify certain responses of connective tissue These may be summarized briefly Cortisol diminishes fibrillogenesis (40 42) and according to some investigators inhibits multiplication of fibroblasts (15) Several authors have reported that this hormone decreases the synthesis of ground sub stance (38 43) Apparently the reduction of synthesis includes both the sulfated and nonsulfated polysaccharides (43) The storage of fat is apparently also moderated by the presence of ACTH and cortisol [re viewed by Engel (28)] In addition to these more gross effects it has been suggested that the moderation of inflammation induced by cortisol is also mediated partially by the effects of this and related antiphlogistic hormones on the fibroblasts (19) In contrast to the influence of these hormones on fibroblasts histiocytes resemble lymphocytes in that they are exceedingly sensitive to cortisol and are destroyed by this hormone when it is present in amounts much smaller than those required to destroy fibroblasts (17 18)

I The Morphological and Histochemical Changes Induced by Adrenocortical Hormones

A major effort in our laboratory has been directed toward the study of the interrelationship of steroid hormone metabolism as it relates to the anti inflammatory effect of the naturally secreted hormones cortisol and cortisone These investigations have led us to the belief that the primary sites of anti inflammatory effect and in addition the metabo lism of steroid hormones are related to the functions of reticuloendo thelial and fibroblastic cells It is now well established that cortisol is a unique and highly potent anti inflammatory agent (24) It exerts its antiphlogistic effect within an inflamed volume of tissue and apparently acts by inhibiting the focal damage of fibroblasts and endothelial cells and in turn preventing the influx of non autochthonous cells into the injured tissue (19 30 34)

The anti inflammatory effects of steroid hormones have been studied by a technique designed to express the fluctuations in numbers of na tive and invading cells of normal and inflamed loose connective tissue (23 24) According to this technique the numbers of cells are ex pressed as per cent/mm² of loose connective tissue The vast majority of cells of normal loose connective tissue (90%) are fibroblasts (24)

When the tissue is inflamed by administering a mild phlogogenic stimulus it may be seen that, depending upon the time after stimulation there is destruction of fibroblasts and an appearance of variable numbers of Pmn s and macrophages (24) Cortisol administration topically or systemically decreases the fibroblastic destruction and the entrance of Pmn s lymphocytes and macrophages (18 19) It has been demonstrated that regardless of the type of inflammatory stimulus the cellular architecture of the tissue is maintained in a partially normal fashion



FIG 1 Rounded up fibroblasts Air dried loose connective tissue spread following s c injection of 25 μg cortisol May Gruenwald Giemsa staining Magnification × 1200

(18 24) The degree of moderation of response to a standard inflaming stimulus is linear over a range of dosages of the hormone (19 24) The anti inflammatory function does not induce an all or none response but rather is a power function in which unit inhibition is a function of dose This generalization of course as in all biological phenomena is manifest only over a particular dose range This technique has been adopted as an assay procedure by Holtkamp *et al* (35) Their data which includes numerous studies including systemic administration of hormones support the generalizations made here

Cortisol given topically (anti inflammatory doses) induces a rounding up or epithelial type change in many but not all fibroblasts whether given to inflamed or control animals (24 26) These cells also acquire a more densely basophilic cytoplasm which contain vacuoles (26) Large doses (1 mg) given topically destroy the local fibroblasts (19) It becomes apparent that the rounded up fibroblast is resistant to the wave of fibroblastic destruction which takes place following application of inflaming stimuli (16 19 24) The suggestion was made that inflammation is potentiated by an autocatalytic process of cell



FIG 2 Radioautographs of loose connective tissue after topical cortisol 4 C^{14} injection Areolar tissue and superimposed film strip were exposed for 8 months and stained with hematoxylin eosin Magnification × 600

destruction which is interrupted because some fibroblasts are resistant to the accumulation of substances which bring about their swelling and lysis (26) A theory which is a physical interpretation of the antiphlogistic potency of hormones based on the interruption of the chain reaction of cellular destruction was introduced by Eyring and Dougherty (30) (See Figs 1 and 2)

Similar preparations of normal and inflamed loose connective tissue were studied by histochemical methods (26) Strips of these tissues

were treated with neotetrazolium triphenyl tetrazolium blue tetrazolium and were also subjected to the Ashbel Seligman reaction (45) These results are summarized briefly here The cytoplasm of rounded up fibroblasts in cortisol treated groups contained markedly increased amounts of reducing substances (26) This could indicate that there is an increase in sugar like substances in the cytoplasm of the hormone treated cells other than the steroid hormones themselves This possibility is strengthened by the fact that reducing substances persisted when cortisol-4 C^{14} could not be detected

Another finding of unknown significance is that frequently many mitotic figures are found among the cortisol treated fibroblasts (24) This finding has not been analyzed to determine whether this is a growth stimulation or is an interruption of mitosis The latter could well be the case since cortisol tends to inhibit wound healing and growth of fibroblasts in tissue culture (34) Since the morphological changes described here have also been found in tissue culture fibroblasts treated with cortisol (34) it appears that this is a direct cellular effect of the hormone and is not mediated through systemic mechanisms Growth inhibition of fibroblasts in tissue culture has also been demonstrated as a direct function of cortisol (32)

II PARTICIPATION OF FIBROBLASTS IN THE SYNTHESIS OF CHOLESTEROL

It has been demonstrated by many individuals that cholesterol is rapidly synthesized *in vivo* from administered acetate For example cholesterol synthesized from labeled acetate has been found within minutes following subcutaneous administration of the precursor (44) The most ubiquitous cell and one long suspected of being concerned with atheroma formation is the fibroblast For this reason the ability of fibroblasts in tissue culture to synthesize cholesterol was investigated in our laboratory By using radioactive 2 C^{14} acetate we were able to isolate C labeled cholesterol after an 8 day incubation of tissue culture fibroblasts (Strain U 79 of Swim) (11 22)

The medium and the cells were saponified and extracted with chloroform Chloroform was evaporated and the residue chromatographed on paper The zone where cholesterol runs in the system was eluted and precipitated with digitonin The digitonide was hydrolyzed and the cholesterol fraction was isolated and divided into two aliquots These two aliquots were crystallized to constant specific activity One aliquot was oxidized the other one was acetylated The derivatives had the same specific activity as the parent compound proving that radioactive cholesterol was synthesized from acetate by the fibroblasts (Fig 3) Thus there seems little question that the fibroblast in addition to

its many other synthetic capacities is a major producer of cholesterol. A future problem of considerable significance is the amount of cholesterol which this cell retains following synthesis The data presented here briefly indicate that since synthesized cholesterol was in the cell wash the tissue culture fibroblast not only synthesizes but can secrete this substance As possibly unknown factors influence the secretion or

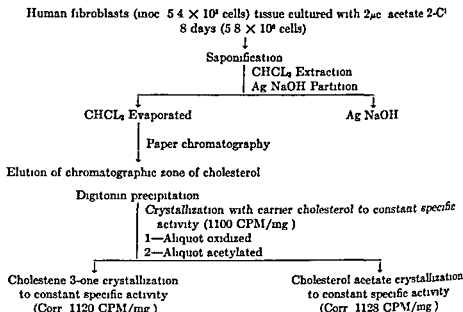


FIG 3 Identification of cholesterol C^{14} synthesized from 2 C^{14} acetate tissue culture fibroblast

retention of the sterol from or in the cell cholesterol deposition could start at focal points by being sequestered in the fibroblast and subsequently following a breakdown of the cell the substance is deposited

III INVESTIGATION OF THE CYTO DEPOSITION OF ADMINISTERED CHOLESTEROL

It is clear from the previous section that cholesterol is synthesized by fibroblasts The cellular sites where this sterol is deposited were also investigated This problem was approached by administering C^{14} labeled cholesterol to rats and ascertaining the tissue and organic sites at which it accumulates (22) A balance study was performed which will be

given in detail elsewhere The data which pertains to the connective tissue studies are described here

Rats were injected with 1 μc of cholesterol-4 C^{14} and were sacrificed at various intervals from 1 hour to 12 days The rats were divided into three main groups Group one included nonoperated nontreated cholesterol-4 C^{14} injected controls group two received the same treatment but was given thyroxin group three was given ACTH From each group the specific activity of the tissues expressed as cpm/mg dried tissue was determined for a great number of organs and tissues

The distribution of cholesterol-4 C^{14} administered intravenously in propylene glycol or serum is given in Fig 4 Only the levels in bloods

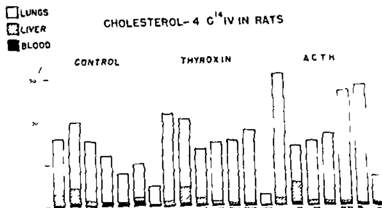


Fig 4 Represents the relative specific activity expressed in cpm/mg of dry tissue from lungs liver and blood after infusion of cholesterol-4 C^{14} i v in rats Three groups are represented controls thyroxin and ACTH treated Upper level for each tissue represented includes the total area from 0 level

livers and the lungs of these rats are considered here Every single rat retained more radioactivity in the lungs than in any other organ or tissue This was also true for cholesterol-4 C given by stomach tube In the controls there was an increase in the lung which was maximum at 6 hours after this it decreased slowly to the twelfth day Animals given thyroxin did not show much difference in concentration of cholesterol in any organs However in the ACTH treated rats there was a tremendous *increase of cholesterol-4* C^{14} *at 1 hour in the lung* after which there was a decrease and then a steady increase On the twelfth day the specific activity of the lungs was higher than those of controls or thyroxin treated rats This indicates that ACTH enhances either the deposition the retention or both of cholesterol

1. *An Extra-Adrenal Cortical Effect of ACTH*

It should be noted (Table I) that the effect of ACTH in enhancing deposition and increasing cholesterol retention is not dependent upon the presence of adrenal cortical secretion. In addition to lung retention it may be of significance that the concentration of cholesterol in the aorta is significantly increased as compared to non-ACTH-treated animals (Table I).

TABLE I

DISTRIBUTION OF RADIOACTIVITY 45 MINUTES AFTER INFUSION OF CHOLESTEROL-4-C14

Tissue	Intact animal			Eviscerated animal
	Nontreated	*ACTH* treated	Adrenex and ACTH treated	ACTH treated
	(cpm/mg dry tissue)			
Lung	186.7	501.0	665.0	560.0
Liver	4.5	10.9	9.4	—
Spleen	7.4	5.3	6.4	—
Blood	1.1	3.2	1.0	3.5
Adrenal	1.9	3.6	—	8.0
Aorta	0	1.4	1.5	3.4
Pituitary	2.5	1.8	1.6	4.4
Kidney	0	0	0	1.3
Heart	0	0	1.0	1.4
Muscle	0	0	0	0
Sm. intestine	2.1	1.3	2.6	0
Testis	0	0	0	1.0
Thymus	0	0	0	1.7
Fat	0	0	1.6	0
Brain	0	0	0	0

Insignificant counts

Although it is premature to assign great significance to this finding *with respect to arteriosclerosis, we feel that it is not without meaning.* Several pertinent findings by a number of investigators also indicate that ACTH has a role in extra-adrenal cortical cholesterol deposition. Severe arteriosclerosis was produced in young mice given daily doses (0.02 mg.) of ACTH for a period of 38 weeks (16). Major lesions were found in hearts, brains, and testes. Recently similar results were reported for rats (49). The distribution of lesions, blood findings for cholesterol, etc., are identical to those shown for mice many years ago

(16) It is significant that it is difficult to produce arteriosclerosis in these species by cholesterol feeding

The recent finding that ACTH produces severe atheromatosis in beagle dogs which were previously made thyroid deficient (39 41) also implicates the role of ACTH in the deposition of cholesterol Beagle dogs are highly resistant to arteriosclerosis on high cholesterol diets which supports further the importance of these findings In addition to the extra adrenal effects of ACTH on cholesterol metabolism Engel (29) has found that ACTH mobilizes fat when given to adrenalecto mized rats These observations cannot help but stimulate the thought that ACTH itself is a major regulator of lipid metabolism throughout the body

IV CELLULAR LOCALIZATION OF ADMINISTERED CORTISOL AND CHOLESTEROL

Cortisol-4 C^{14} was given subcutaneously to adrenalectomized mice and strips of treated subcutaneous tissue were removed at intervals and radioautographed (26) The cellular localization of the labeled cortisol in connective tissue was thus determined The fibroblasts either contained the hormone or it was located at their surface Histiocytes and lymphocytes did not concentrate the hormone Fat cells contained little By chance several connective tissue preparations contained a few muscle fibers and axons There was a considerable amount of radio activity in the myelin sheath and on the sarcolemma The muscle cell itself did not appear to contain sufficient amounts of hormone to expose the emulsion As qualitative as such investigations are they do indicate that the vast amount of radioactive hormone in connective tissue was concentrated by fibroblasts (see Fig 2)

Although all tissues were not radioautographed due to technical problems in the cholesterol-4 C^{14} treated animals the amount of radio active cholesterol in the lung made it possible to obtain radioautographs of this weak β ray emitter Cholesterol was almost exclusively found in the reticuloendothelial cells of the lung (in both interalveolar and peribronchial histiocytes) (Fig. 5) The epithelial cells were not found to contain it None was found in small blood vessels

It is apparent that whereas cholesterol is readily taken up by the RES fibroblasts do not avidly ingest administered cholesterol under the conditions of the present experiment It is possible that cholesterol when combined with some other substances may enter the fibroblasts This possibility is now under investigation Cortisol appears to be con centrated by fibroblasts to such an extent as to be considered a function of this cell

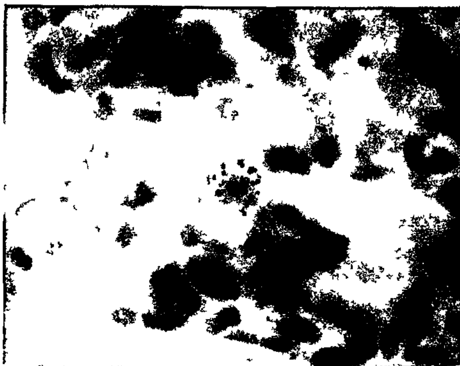


FIG 5 Radioautograph of lung following intravenous cholesterol-4-C^{14} administration The black dots in the macrophage are typical β tracks Note absence of radio activity in epithelial cells Frozen section superimposed film strip exposed for 6 weeks Hematoxylin and eosin Magnification × 600

V METABOLISM OF CORTISOL BY CONNECTIVE TISSUE CELLS

The facts given above indicate that many functions of cortisol are mediated by the action of this hormone on fibroblasts For this reason it seemed worthwhile to investigate the ways fibroblasts in turn influenced the metabolism of cortisol The results of these studies yield information applying to the mechanism of hormone action at the cellular level and secondly yield information concerning the metabolism of cortisol in the tissue where it appears to exert its greatest influence This local metabolism in turn of course determines the over all amount of the altered molecules found in blood which is eventually excreted This subject will be touched upon briefly below

1 *Micromethods Used for Steroid Identification and Quantification*

In order that the problems stated above might be approached micro methods were designed by Berliner and Salhanick (6) To a large extent the methods used are classical chromatographic methods of Zaffa

roni (50) Microchemical techniques developed in our laboratory are outlined briefly here and given in detail in other publications (2 9)

Ring labeled steroids were identified by using the Zaffaroni chromatographic procedure A derivative which would tag or make radioactive steroids was also employed We have used 1 C^{14} acetic anhydride as shown in Fig 6 Acetic anhydride will attach itself to an

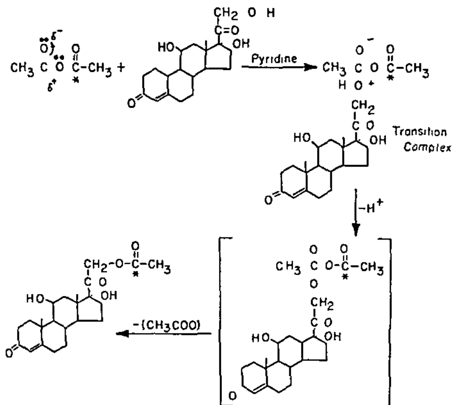


FIG 6 The quantitation and identification of cortisol by acetylation with radio active 1 C^{14} acetic anhydride and pyridine

acetylable OH group Thus a radioactive derivative of the steroid is formed (2) In Fig 6 the electronic configurations of the acetic anhydride and the acetylable OH group is represented A transition complex is formed to fulfill the octet of C I

By this method we are able to identify the steroids using chromatography In addition since there is 100% acetylation the amount of radio activity would indicate the amount of acetylated steroid Therefore

identification and quantification are made possible. The sensitivity of the technique is in fractions of a μg. Additionally, already labeled (4 C^{14}) steroids may be acetylated with 1 C^{14} acetic anhydride and are thus doubly labeled. This procedure makes the technique even more sensitive. The use of these techniques should be of great importance in both clinical and experimental medicine since they allow the quantification of steroids in small amounts of body fluids and tissues (10).

Normal loose connective tissue of mice (0.3–0.6 gm.) was incubated in the presence of cortisol-4 C^{14} in a phosphate buffer pH 7.4, 37°C for 3 hours (4). The loose connective tissue very actively metabolized cortisol to various compounds (Fig. 7). These were: 20-epi Substance

FIG. 7. Radioactive tracing from paper chromatogram obtained from the incubation of cortisol-4 C^{14} with loose connective tissue.

E of Reichstein (reduction form of cortisol in the 20 position), dihydrocortisol (the reduction of the double bond in the 4 position), cortisone (removal of hydrogen from the 11 position), corticosterone (removal of hydroxyl group at the 17 position), and 11 β hydroxy Δ^4 androstenedione (the cleavage of the side chain).

From these *in vitro* (and also *in vivo*, *vide infra*) studies it may be seen that the only changes we can find in a molecule of cortisol are oxidations and reductions of substituted groups on the gonane nucleus. The gonane nucleus is not metabolized or utilized, that is, there is no breakage of the rings or utilization of its carbon atoms. Since 90% of cells of loose connective tissue are fibroblasts and it seems unlikely

that a few other cells could perform all these conversions it appears that the fibroblast is the main site of metabolism of cortisol However to ascertain precisely whether this is the case pure lines of human tissue culture fibroblasts were used to study their metabolism of cortisol 4-C^{14} Tissue culture fibroblasts were treated with cortisol-4-C^{14} and products of their metabolic activity were isolated (47) Again these cells were able to reduce the 20 position of cortisol to form Substance E cortisone corticosterone and 11 β hydroxy Δ^4 androstenedione The one significant difference was that the tissue culture fibroblasts formed primarily the 20β form of Reichstein s E and only small quantities of the 20 epi isomer The differences found were quantitative The amount of cortisone formed by the tissue culture fibroblasts was not the major product of conversion as compared to that for loose connective tissue The C 19 compound 11 β hydroxy Δ^4 androstenedione was found in greater amount in the tissue culture preparation than in loose connective tissue Inflamed connective tissue incubated in the same way did not convert cortisol to other compounds (5) The most likely reason for this is that in incubations of the same amount of inflamed connective tissue there were fewer fibroblasts since so many of these cells were destroyed If some compounds were present they could not be detected by these methods

2 *Malignant Connective Tissue Cells*

Fibrosarcoma cells (S 37) of mice and osteosarcoma cells of tumors of dogs were incubated (48) It was found again that cortisol can be converted by the malignant counterparts of normal cells to cortisone to Substance E of Reichstein to Substance U of Reichstein to corticosterone and to dihydrocortisol and 11 β hydroxy Δ^4 androstenedione However one of the metabolites which was produced in large amounts was 11 keto etiocholanedione a compound which was not observed or isolated from the incubates of normal connective tissue or from the tissue culture fibroblasts Other investigators (33) found that a strain of fibroblasts which are resistant to the growth inhibiting effects of cortisol metabolize 100% more cortisol than a cortisol sensitive strain (U_1 79) Similarly several years ago it was found in our laboratory (8 20) that lymphatic leukemic cells which are poorly susceptible to lytic effects of cortisol are able to metabolize this hormone at a much greater rate than normal lymphocytes which are extremely sensitive to cortisol induced lysis It is also interesting to note that like the malignant fibroblastic cell leukemic lymphocytes very actively convert cortisol to 11 β hydroxy Δ^4 androstenedione

VI Products of Cortisol Metabolism in Blood and Urine

The products of steroid metabolism found in blood and urine are formed by two large pools of cells These are the cells distributed throughout the body which might be for convenience called the body tissue Since certain products of metabolism are unique to liver cell function these may be termed hepatic tissue products Each one of these groups of metabolic functions influences the other as to rate of metabolism and persistence of hormone function

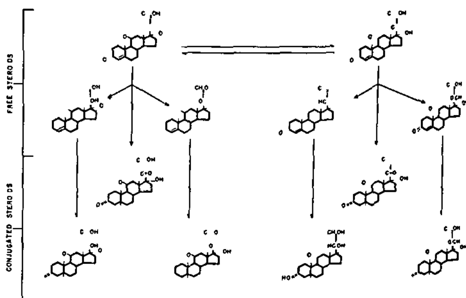


Fig 8 The major products of transformations by extrahepatic tissue are those represented as free steroids note that all have the α β unsaturated ketone In intact animals all the products represented in this figure have been isolated and identified after the infusion of cortisol or cortisone

The general tissue cells appear to metabolize cortisol to products which are identical to those produced by fibroblasts in loose connective tissue and tissue culture *vide supra* Although it may be that certain organs such as brain may yield certain unique products for that organ the aggregation of metabolites is still no different than those produced by connective tissue Berliner *et al* (12) have shown that in hepatecto mized rats cortisol-4 C^{14} is converted to substance E of Reichstein sub stance U of Reichstein cortisone dihydrocortisol and 11 β hydroxy Δ^4 androstenedione (Fig 8) Animals from which the livers spleens

adrenals kidneys and ovaries were removed were given cortisol-4 C^{14} and once again the same conversion products were found

Intact rats given 4 C^{14} cortisol and studied in the same manner (whole body extraction) had the conversion products listed above but in addition the tetrahydrohomologs of each of these compounds were isolated in the conjugated form (see Fig 8) It appears then that the unique function of the liver is the ability to reduce the 3 ketone and conjugate the metabolites of cortisol Berliner and Weist (7) have also demonstrated that the liver is essential for 3 keto reduction and conjugation of progesterone-4 C^{1}

It is premature to assume that under normal conditions the fibroblasts are the sole site of extrahepatic cortisol metabolism However since they are probably the most ubiquitous and numerous cells in the organism they must contribute enormously to the pool of steroid metabolites Certainly among normal connective tissue cells fibroblasts alone appear to be able to perform these steroid conversions since lymphocytes (20) and inflamed connective tissue (which contains histiocytes and polymorphonuclears but few fibroblasts) were devoid of the capacity to perform these metabolic functions

1 *Inhibition of Steroidal Conjugation by ACTH*

Experiments both *in vitro* and *in vivo* in mice rats and humans indicate that ACTH in the absence of the adrenal gland is able to inhibit steroid conjugation by hepatic cells (21) This work has not as yet been published in detail In general the experiments show that the livers of stressed mice cannot conjugate steroids normally *in vitro* Similar inhibition is produced when livers of normal mice are treated with ACTH and cortisol-4 C^{14} or corticosterone 4 C^{14} *in vitro* Adrenalectomized mice and rats were given 4 C cortisol and the half life was determined When ACTH was given before or during the course of cortisol treatment the half life of the hormone was markedly prolonged Similar results were found for humans given cortisol-4 C when the same individuals received ACTH in addition to the radioactive hormone Reports in the literature indicate the importance of stress as a factor in prolonging the half life of circulating cortisol (31 37)

Thus ACTH appears to enhance the blood level of cortisol by inhibiting its excretion as well as by increasing its secretion

VII Metabolism of Cortisol-4 C^{14} in Normal and Inflamed Animals

Radioactive cortisol was given intravenously to adrenalectomized mice which were sacrificed at intervals (27) Samples of blood and

various tissues were taken at 10 20 30, 60 and 100 minutes after the injection The blood and various tissues were counted extracted, chromatographed and the steroids in each were identified Analysis of the blood showed that cortisol is very rapidly transformed to tetra

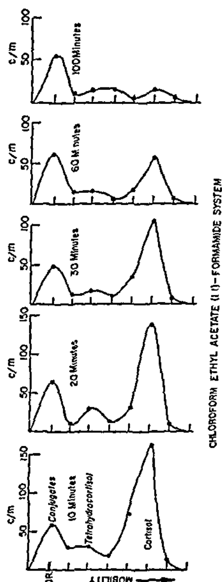


Fig 9 Representation of samples of blood at various time intervals after the administration of cortisol-4 C^{14} Note the disappearance of cortisol in the lower portion in relation to time

hydrocortisol and conjugates (Fig 9) The half life of cortisol in mice is very similar to that of humans i e from 45 to 55 minutes (3) When the percentage of radioactivity from the different chromatographic peaks of the blood were plotted we found that the free cortisol decreases and in from 40 to 60 minutes is equal in amount to the conjugates after which it disappears from blood at 100 minutes (Fig 9) At 10 minutes the conjugates increase and free cortisol decreases as it is conjugated and excreted (Fig 9) However tetrahydrocortisol remains constant from 10 to 100 minutes This indicates probably that cortisol is being transformed to tetrahydrocortisol and tetrahydrocortisol is then conjugated The conjugate was separated and identified by paper chromatography

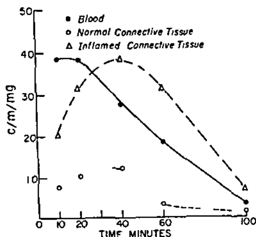


FIG 10 Representation of the relative specific activity expressed as cpm/mg of dry tissue Blood normal connective tissue and inflamed connective tissue at various time intervals after the infusion of cortisol 4 C[14]

Adrenalectomized mice were inflamed by subcutaneous injection of 1% gelation on one flank and not on the other Thus normal connective tissue from each mouse was the control for the opposite side The normal connective tissue the inflamed connective tissue and the blood were plated and the relative activity (cpm/mg) was determined at different intervals after the injection

The relative specific activity of the inflamed connective tissue increases to a maximum at 40 minutes after which it decreases and follows the same curve for diminution as the blood (Fig 10) At 40 minutes the relative activity of the inflamed connective tissue is higher than the relative activity in the blood or in the normal connective

tissue Normal connective tissue and inflamed connective tissue follow the same curve of disappearance However normal connective tissue contains less radioactivity This is most likely due to the fact that in flamed tissue tends to accumulate blood constituents in a nonspecific manner Apparently there is no specific trapping mechanism for cortisol in inflamed areas

After paper chromatography of both normal connective tissue and inflamed connective tissue we did not find any qualitative differences in the compounds present in these two tissues The conjugates tetra hydrocortisol and cortisol are present in both tissues in the same ratios again proving that there is no special mechanism that would trap cortisol specifically in the area of inflammation There are compounds which do not have anti inflammatory effect and which also concentrate in zones of inflammation such as vital dyes The presence of tetra hydrocortisol—a product of the liver only—indicates that an equilibrium with blood steroids is operative and is nonspecific The metabolites of cortisol produced by connective tissue which we have isolated and studied here have been tested for antiphlogistic activity and none have any higher degree of antiphlogistic activity than cortisol (24) Cortisol is the most potent antiphlogistic corticosteroid normally produced (24)

VIII General Discussion

The interests of numerous investigators in metabolism of steroid hormones and sterols has been focused on the function of the liver in metabolizing these substances The data reviewed here indicate that many changes in steroid *molecules* are produced by other than hepatic cells The weight of evidence indicates that fibroblasts are very potent transformers of the steroid molecule whereas reticuloendothelial cells do not appear to be able to perform these same functions On the other hand certain sterols seem to be sequestered by histiocytes

The functional meaning of steroid transformations in connective tissue is an intriguing problem At this time one can only speculate as to their importance for hormone action However certain facts appear now to be sufficiently verified to support some concepts

It has been assumed that conversion of steroid hormones is a degradative process because the metabolites are physiologically less active however it has not been taken into consideration that the process of oxidations and reductions which take place during the transformation could be the way in which the hormone exerts its function The gonane nucleus is synthesized by the body but it is not catabolized in the sense that its carbon atoms are utilized in intermediary metabolism Rather it is excreted after the oxidations and reductions of the sub

stituted groups In general although some carbon atoms of cholesterol do enter general metabolism (side chain) the problem of ridding the body of its gonane nucleus is also an excretory one Thus the turnover of steroids does not represent the utilization of the molecule as such but comes about as an alteration in its substituted groups and by an excretion of the skeleton (gonane nucleus) which carries them Thus transformation in molecular structure represents exchange of protons and electrons with subsequent conjugation and excretion of the ring structure

The problems which face the physiologists are two those related to the actual function of a particular configuration of a steroid molecule in a particular process and those related to the conjugation and excre tion of the carrier When cortisol is "utilized in a physiological sense it is being converted through oxidation and reduction to a molecule which no longer possesses the function of cortisol itself It is suggested that the process of conversion then is in part a mechanism of cortisol action It appears that some functions such as the anti inflammatory effect and gluconeogenesis in the liver continue to take place when cortisol as such is no longer present (as measured by the presence of the ring labeled steroid (36) It was found that the anti inflammatory effect persists for hours after radioactivity from the 4 C^{14} cortisol has gone from inflamed connective tissue (27) Thus the transformation of cortisol is rapid (3) its effects are produced gonane nucleus is conjugated and it enters excretory mechanisms On the other hand the lymphocytolytic effect of cortisol is a function of the hormone itself (20) At least no measurable conversion products are found

It appears then that when cortisol participates in influencing cer tain metabolic functions of connective tissue cells the processes of its conversion are a part of its function and in the case of the fibroblast it leaves the cell in an altered state which resists destruction Other cells such as macrophages and lymphocytes are destroyed by cortisol in infinitesimal amounts without biochemical modification of the mole cule

Cholesterol has a longer side chain and does not have hydroxyl groups in any other place except the 3 position Cortisol is a C 21 steroid with substituted hydroxyl groups at the 11 17 and 21 posi tions These differences influence the different half lives of these com pounds in the body Cortisol is a very active and rapidly metabolized steroid in contrast to cholesterol which is a non or very poorly metabolized steroid Another factor which may make a difference in half lives is that cortisol and its conversion products are mobilized in the liver very rapidly Thus it is delivered to a site where it is rapidly conjugated and excreted However the lung is not an organ which will

metabolize cholesterol to any great extent (46) It retains it there for a long period of time and releases it slowly through the blood Since the cholesterol released by lung must enter the circulation through the pulmonary veins its concentration should be great in the left heart and coronary arteries and aorta It has been shown in our laboratory that ACTH will also induce cortisol as well as cholesterol retention ACTH acts on the liver and inhibits conjugation of the steroids (21) Thus the level of cortisol in blood is the result of two factors an increased output of this steroid by the adrenal gland and a decreased rate of conjugation by the liver In the case of cholesterol there is also greater retention but this appears to be due to a greater degree of sequestration of the sterol by reticuloendothelial cells Thus cholesterol retention can take place without a hypercholesterolemia It could be that the reticuloendothelial cells of the body form a buffer which protects the organism against widespread deposit of poorly metabolized sterols

It should not be assumed that the authors feel that the only site of cholesterol phagocytosis is in the lung In these studies the concentration was so great it could be measured and autographed with ease The suggestion that the reticuloendothelial cells take part in cholesterol metabolism is very old and considerable literature has developed on this subject (review 14 25)

Recently it was concluded (14) that the participation of the Kupffer cell of the liver is necessary to the disposition of ingested cholesterol These authors came to this conclusion by observing the deposition of administered radioactive cholesterol in various organs including the liver It has been pointed out that C^{14} cholesterol given in the form of lymph chylomicra leaves the blood stream after administration within 5 minutes of its entry and again appears in the blood as soluble lipo protein cholesterol in 10 minutes (13) This removal rate is highly suggestive of rapid reticuloendothelial phagocytosis The role played by the reticuloendothelial cells in the metabolism of the cholesterol molecule is as yet unknown However the fact that free cholesterol is rapidly phagocytized by these cells and that in turn reticuloendothelial cells and their derivatives are suspected of forming a portion of the plasma proteins particularly β and γ globulin leads one to the attractive hypothesis that reticuloendothelial cells are concerned with the protein binding of cholesterol

ACKNOWLEDGMENTS

The research represented by the foregoing manuscript has been aided by the following grants Army Contract #DA-49 007 MD 130 U S Public Health Service Grant No CY 2349(C4) and American Cancer Society Grant No T 32 The authors gratefully acknowledge this assistance

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Discussion

CHAIRMAN HARTROFT You mentioned that edema in the connective tissue had to be considered but how about the rate of blood flow in inflamed connective tissue particularly in the initial stages of the inflammatory reaction where it is much greater? Could this possibly explain why more cortisol reaches the inflamed tissue and develops there simply because there is more blood flow there and more permeability? At the end was the cholesterol injected?

DR BERLINER Yes it is our feeling that the increased cortisol concentration is a direct result of the edema Cholesterol was injected into the saphenous vein of each of the rats used

CHAIRMAN HARTROFT Would you get the same result if you gave it orally

DR. BERLINER Yes the result is the same but the differences in the relative specific activities are not as pronounced However the lungs always exhibit the highest relative specific activity and the liver the second highest These results were observed in a 15 hour experiment where cholesterol was given intragastrically When J H Bragdon and R S Gordon Jr (*J Clin Invest* 37 574 1958) inject fatty acids into rats they find fatty acids concentrated in the liver but 200 minutes later they find the highest concentration in the lung measuring relative specific activity just as we have done

If we determine endogenous cholesterol per gram of tissue we find a higher concentration of this sterol in the lung than in the liver Therefore if the specific activity of the cholesterol-4 C^{14} injected was determined after isolating it from the lung and the liver the specific activity would probably be lower in the lung This of course would reflect dilution of the isotope labeled sterol by endogenous cholesterol

DR WEXLER I think it would be interesting to test the antagonistic effects of the glucocorticoids

DR BERLINER Mineralocorticoids such as desoxycorticosterone do not have any antiphlogistic effect Furthermore they do not affect fibroblast morphology in the same manner that cortisol does Dr Dougherty has done quite a bit of work on this problem

DR. MEYER Did you use tetrahydrocortisone in the eviscerated rat? Is this not conjugated to either glucuronic acid or sulfate?

DR BERLINER That is what is going on in our laboratory now I agree that it is a very important thing to do because the liver first reduces steroids at position 3 and then conjugates But we do not know whether conjugation will occur with 3 hydroxylated steroids in extrahepatic tissue

DR ADLERSBERG You mentioned that cortisone has maximum antiphlogistic effect but from experience we know that some derivatives of cortisone or hydro cortisone are more powerful milligram for milligram for example prednisone and prednisolone

DR BERLINER Our experiments deal with natural products of the adrenal gland and not with synthetic compounds like prednisolone

CHAIRMAN HARTROFT In the second microphotograph that you showed of the tissue cells would you consider these in an irreversible stage? To me the nuclei in those cells looked a little bit hyperchromatic compared to one in the photograph

DR BERLINER Dr Dougherty demonstrated this effect on fibroblasts for the first time in 1949 It has also been shown with tissue cultures of fibroblasts by Swim and by Holden and Adams The cells in tissue culture are in the presence of cortisone rounded up in the same way as in loose connective tissue It is re versible

DR WHITE You stated that excretion of cortisol has occurred at an early stage and yet subsequently one still has evidence of the anti inflammatory action of the steroid. Have you established the rate at which the labeled steroid leaves the fibroblast as well as actual proof that the steroid has entered the fibroblast?

DR BERLINER Yes We chromatographed extracts of inflamed and noninflamed connective tissue at various intervals up to 120 minutes At 120 minutes we could not detect any radioactivity A similar finding has been described by Hyde for the gluconeogenic effect of cortisol showing that when the hormone is no longer present the gluconeogenic effect is maximum at a later time

DR. MOON I would like to ask what concentrations of cortisol were used in tissue cultures?

DR BERLINER We used 0 3 μg per milliliter of nutrient media

DR JEANLOZ What was the evidence for the conjugation at position 3?

DR BERLINER The eviscerated rat does not conjugate the steroid nor does it reduce the 3 position However in the intact rat both of these events take place We isolated the conjugated steroid and acetylated it Then we hydrolyzed the con jugate and found the 21 monoacetate of the compound

DR JEANLOZ You hydrolyzed the group at position 3 without removing the acetyl at position 21?

Dr Berliner Yes we did an acid hydrolysis

Dr Riegel Dr Berliner how quantitative was the over all recovery of the microcuries of the radioactive cortisol?

Dr Berliner We developed a method for quantitation and Mr Westenskow constructed in our laboratory a strip counter of high sensitivity for the determination of radioactivity We can recover from 85 to 95% of the injected material

Dr Adlersberg In connection with the high concentration of radioactivity in the lungs after injection of cholesterol if I am not mistaken you have also some histological evidence of greater accumulation of this substance in the lungs

Dr Berliner Dr Dougherty presented this data at another conference and he showed foam cells in the lung containing cholesterol C Seeman (*Beitr pathol Anat u allgem Pathol* 83 705 1930) and C Cioni (*Sperimentale* 86 587 1932) showed similar results

Chairman Hartroft Have you looked at lymph nodes?

Dr Berliner No

Chairman Hartroft I think you would find something there The idea that it goes only to the liver is I believe fast on its way out

Elastic Tissue in Atherosclerosis

ALBERT I LANSING

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Pittsburgh Pennsylvania

I assume that I am here at this conference because I have held for many years that alteration in the elastic tissue bed of arteries is an age dependent precursor of human atherosclerosis Over the years we have attempted to study the structure and chemistry of the elastic tissues Our thesis has been that changes in the arterial wall create a receptive environment for lipids in the intima At least in the human the most common change in the arterial wall conducive to lipid deposition in the intima is a breakdown of the elastic bed either in the elastica interna, elastica externa or the elastic lamellae of vessels like the aorta

The working hypothesis that alteration in the architecture of the arterial wall is a preliminary step to atheromatosis is consistent with the well established observation that luetic aortitis is accompanied by an aggravated level of atheromatosis In luetic aortitis there is extensive damage to the media of the aorta with resultant replacement of elastic lamellae and smooth muscle by scar tissue In further support of this suggestion is the fact that the pulmonary artery of the human is normally resistant to atherosclerosis This vessel we have established is also resistant to the age dependent breakdown and calcification of elastic tissue that typifies most other arteries

Clearly to attribute atherosclerosis to no more than a disturbance of lipid metabolism is to grossly oversimplify the problem There is good reason to believe that receptivity of the arterial wall is as consequential as disturbed lipid metabolism in the genesis of atherosclerosis

In the discussion to be offered I plan to recapitulate in a general way some of the early studies to our current experimental approach to atherosclerosis Time will not permit a full discussion of the structure and chemistry of elastic tissue or more than a superficial introduction to the crude experiments on the systemic effects of elastase

Purely for purposes of introduction it may be worth while to recall a few features of atherosclerosis that too often are forgotten or shoved under the rug for the sake of expediency

First it might be well to recall that atherosclerosis is not a human disease In its spontaneous form it occurs in a wide variety of species

including the chicken pig bear baboon otter skunk sheep and many other species that have been examined

Secondly atherosclerosis is not a new disease in the history of the human species It dates back as far as material has been available for analysis Ruffer in his excellent volume has illustrations of atherosclerosis histologically identical to that seen today but prepared from mummies of Egyptian priests The latter according to records were not subjected to stress and were largely vegetarian It appears clear that the professional men of today the physicians and hard pressed business men have no priority on atherosclerosis

I think we also know rather clearly that atherosclerosis begins early in life perhaps in the second decade and that the disease progresses steadily both in frequency and severity as a function of age This point is supported by the original work of Blumenthal (1) in 1944 that opened our interest in human atherosclerosis

A total of 642 human aortas obtained at autopsy were examined in this study Hematoxylin and eosin staining and microincineration were used in combination to evaluate the frequency of occurrence and the influence of age sex and disease on calcification of the medial elastic tissue of the human aorta The results strongly indicated that calcification of the media precedes formation of intimal plaques that medial calcification occurs more frequently than intimal plaques that intimal plaques do not occur without calcification of the elastic tissue of the media or other medial damage such as syphilitic aortitis and that in a single aorta medial calcification appears to be more intense in the immediate vicinity of an intimal plaque than elsewhere The calcification of the elastic tissue in the media occurred as a function of age and was independent of sex and various infectious diseases but was intensified by the presence of hypertension

Let us continue to enumerate some of the features of atherosclerosis that are generally recognized There will be no quarrel with the statement that atheromatosis is almost invariably associated with intimal thickening and that this intimal thickening is a function of accumulation of collagen fibers with occasional fibroblasts and macrophages That fat tends to deposit in and around collagen appears to be the case but it is not clear whether or not the fibrosis of the intima precedes lipid accumulation

We have adopted Karsner s view that the sequence of lesion severity is from the fibrous to the fatty and calcified plaques In further extension of this seriation we have proposed that in both elastic and muscular arteries of humans deterioration of the elastic lamellae as evidenced by calcification of and fraying and fragmentation of the elastic lamellae

almost invariably occur atheromata and precede the formation of the fibrous thickening of the intima

The seriation of intimal plaques from intimal fibrosis through fatty plaques and calcified necrotic plaques was substantiated by the data of Yater several years ago in his analysis of the age incidence of the various plaques in human coronary arteries The maximal incidence of fibrous plaques occurred between 18 and 29 years of age and decreased sharply in later years The fatty plaques had a low frequency of occurrence in the early years reached a peak in the mid thirties and decreased in incidence in the late thirties and forties Necrotic plaques showed a minimal incidence early in life and increased steadily with age

A similar conclusion may be reached from the data we obtained by calcium and cholesterol analyses of isolated intimal plaques in human aortas A series of plaques of various grades were carefully excised from the aortas of young and old individuals and analyzed for calcium and cholesterol

TABLE I

ANALYSES OF ISOLATED PLAQUES (INTIMA) GRADED BY SEVERITY AND AGE GROUP AT AUTOPSY SHOWING THAT LESIONS OF SIMILAR GROSS APPEARANCE MAY DIFFER CHEMICALLY DEPENDING ON AGE OF THE INDIVIDUAL

Severity of Lesion	Per cent calcium		Per cent cholesterol	
	Age 30-59	Age 60-80	Age 30-59	Age 60-80
Normal	0 17	1 54	1 9	3 2
Fibrous	0 61	1 62	8 5	24 8
Fatty	6 62	6 17	29 0	29 3
Necrotic	5 56	10 90	47 8	24 5

On fat free dry weight basis

The essential point is that the chemical composition of grossly similar plaques changes with age Fibrous plaques become richer in cholesterol fatty plaques remain similar but necrotic plaques lose cholesterol and gain in calcium content

Still another series of analyses support this view Using aortic tissue from individuals 30–59 years of age a graded series of intimas from normal through fibrous fatty and necrotic plaques were excised After isolating the appropriate areas the adventitia was separated by blunt dissection and discarded The media was carefully separated from the intima and calcium analyses were conducted in the medial tissue while cholesterol analyses were performed on the intimal tissue Figure 1 il

lustrates that the cholesterol content is minimal in normal intimas and climbs steadily from the fibrous through the fatty and necrotic plaques On the other hand the elastic tissue calcification in the media reaches a maximum in the transition from the normal intima to the fibrous plaque

It seems that we are dealing with two temporally and quantitatively distinct phenomena The calcification of the elastic tissue in the media appears to be related to the transition of normal tissue to the fibrotic intima while the cholesterol accumulation becomes striking in the transition from fibrous to fatty plaque

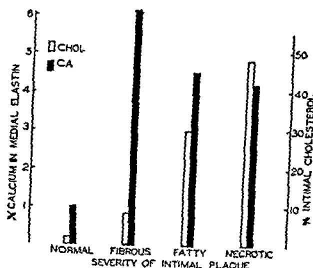


FIG 1 Histogram of calcium and cholesterol analyses in a series of plaques graded by severity Calcium is expressed as percentage in dry defatted medial elastin cholesterol as percentage in dry intima On the basis of this seriation calcium in the media appears to increase before cholesterol From Lansing *et al* (6)

All of this of course is little more than working hypothesis Breakdown of the normal architecture of the arterial wall may predispose to intimal fibrosis and lipid accumulation More data are needed to establish or refute this possibility Some support to this idea is gained from examination of syphilitic aortitis which involves first, the inflammatory reaction then extensive scarification of the media with dissolution of the elastic lamellae This lesion is interesting because it exemplifies disruption of the arterial wall and is associated with severe atheromatosis It seems to me that we are missing the boat in not using syphilitic aortitis as an experimental means of analyzing the genesis of atherosclerosis In an appropriate experimental animal this is a phenomenon

that could well be explored in working out the relationship of the arterial wall to lipid deposition in the intima To my knowledge this has not yet been done and the fact that syphilitic aortitis is accompanied by severe atheromatosis is only casually referred to in the literature

A possible clue to the role of the arterial wall in the metabolism of the intima may exist in the structure of elastic lamellae The point here is that elastica interna or externa or the elastic lamellae of an elastic artery are not fibrillar they are plates If we look down frontally upon an elastic lamella we find that we are dealing essentially with a continuous membrane which consists of elastic fibers of the dimensions of those we see in ligamentum nuchae which are plastered down upon a feltwork of delicate elastic fibers having dimensions almost of the limit of visibility in a light microscope All of these are cemented together in an amorphous matrix material which stains like elastic tissue The result is a continuous sheet with only widely dispersed fenestrations or openings which permit the free movement of materials between the intima and the media Between the fenestrations the lamellae may well be impermeable This description is applicable to arteries from young individuals

The situation is quite different in arteries muscular or elastic of middle aged or old individuals With the calcification and fragmentation of the elastic lamellae as generally found in human atherosclerosis there is almost complete removal of the elastic plates To a large extent arteries may be considered to be relatively avascular organs The vasa vasorum appears to extend no further than the outermost third to half of the arterial wall At least the innermost half is essentially avascular The point is that a breakdown of the elastic lamellae may sharply alter the movement of materials from the adventitia towards the interna and of course in the reverse direction Purely as a speculation one might propose that the breakdown of the elastic lamellae may result in a freer diffusion of materials including lipids through the arterial wall This is at least one way to rationalize the parallel occurrence of both elastic tissue breakdown and atheromatosis in human atherosclerosis

Another point that should be made is that while we generally associate elastic tissue damage and atheromatosis to arteries these phenomena are also found in other vessels Thus the veins of the extremities in elderly subjects can be demonstrated to contain fraying and fragmentation of the elastica and atheromatosis that is indistinguishable from its counterpart in arteries This lesion is referred to as phlebosclerosis

Further Dr Priman and I have recently been studying lymph vessels from forty odd cadavers and it appears that at least the thoracic duct

also is subject to elastica breakdown and atheromatosis The thoracic ducts were removed from cadavers during dissection by medical students and were then routinely stained with H and E and orcein for elastic tissue It is remarkable that tissues of cadavers after preservation for years still stain quite well and preserve many of the histological details The thoracic duct from a young individual is a thin walled structure whose media consists almost entirely of smooth muscle in longitudinal and circular bundles Elastic fibers are virtually absent in the media The endothelium rests directly upon the elastic interna with no apparent subendothelium The thoracic duct of an old individual shows variable but consistent fraying and fragmentation of the elastica intima development of a subendothelium rich in collagenous tissue atheromata and invasion of the media by elastic fragments from the disrupted elastica interna The lesions of the thoracic duct are mild in comparison with those of arteries but nevertheless are recognizable The curious point is that the lesions should exist at all in a vessel that possesses an extremely low intra luminal pressure

Age dependent deterioration of elastic tissue is not restricted to blood and lymph vessels If one examines the stroma of the lung of old individuals one finds extensive fraying and fragmentation of the elastic fibers with a dispersion of the elastic material which gives the impression of a great increase in the amount of elastica present Upon micro incineration and dark field examination of the preparations of lung there is evidence of extensive mineralization of the elastic elements in the stroma

Senile elastosis is an age related lesion of elastic tissue in the dermis which does not conform to the pattern described for vessels and lungs It is a lesion of elastic tissue of the pars papillaris of skin that is chronically exposed as above the collar line and beyond the cuff line In the young individual the elastic fibers of the pars papillaris consist of a very delicate network of fibers that are calcium free In senile elastosis there is a massive accumulation of coarse elastic tissue which becomes the predominating component of the pars papillaris Unlike the elastica of old arteries the material in senile elastosis is basophilic as evidenced by an affinity for toluidine blue Senile elastosis is also distinctive in that the proliferated elastic tissue does not show evidence of calcification when examined after microincineration Although there has been some dispute as to the identity of the pathological fibers of senile elastosis the evidence seems clear that these are elastic or elastic like fibers Incubation with elastase of sections of skin manifesting senile elastosis results in total dissolution of the fibers concerned This can be accomplished by incubating at room temperature at pH 8 4 for a period of 45 minutes

Thus far the data concerning age dependent alterations in the elastic elements of organs have been based upon histological and histochemical procedures Confirming data are obtained if we leave visual techniques and go to analytical procedures

Elastin can be prepared from human aortas by a simple technique which preserves the morphology tinctorial specificity and elastase digestibility of native elastic tissue The intima and adventitia of fresh aorta is stripped by blunt dissection and the separated media is defatted by refluxing with methanol and acetone serially for 1 hour each The defatted tissue is then exposed to 0 1 *N* NaOH at 98°C for 45 minutes and washed The resultant material is defined as elastin is carbohydrate free and preserves all the usual tinctorial and physical properties of elastic tissue

Analyses of the calcium content of medial elastin in human aortas show the appearance of calcium in elastin at the approximate age of 20 years The concentration of calcium in elastin climbs sharply after 20 years to a plateau of 6–8% which is reached at roughly 50 years of age The aortas used in this series of analyses were not selected for condition of the intima To avoid the possibility that the state of the intima might determine the amount of calcium in the underlying medial elastic tissue another series of analyses were performed In this series only plaque free areas of aortas were selected for the several age groups The data show an absence of calcium in elastin at birth about 0 2% calcium in elastin at 20 years (data calculated on a dried, defatted basis) and a steady climb in the amount of calcium with age after 20 years to a level of about 5% (Fig 2)

As noted earlier the pulmonary artery is characterized by a resistance to atherosclerosis except in the case of pulmonary hypertension If the analytical procedures outlined are applied to an age series of human pulmonary arteries it is interesting to note that the increase with age in calcium content of the medial elastin is minimal—in a total of 106 analyses at various ages all but two of the calcium values were at or under 1% The two exceptions one at 2% calcium and the other at 3% calcium represented two 69 year old individuals both of whom had histories of pulmonary hypertension (Fig 3)

Not too much can yet be said concerning the nature of elastase and its systemic effects There has been little substantiation of the work that we have done in connection with the systemic effects of elastase The work from several laboratories has not confirmed our work but there have been two studies that do support our findings It is obvious that more work must be done Progress is hampered by lack of understanding of the structure and chemistry of elastin and the origin of and function of elastase

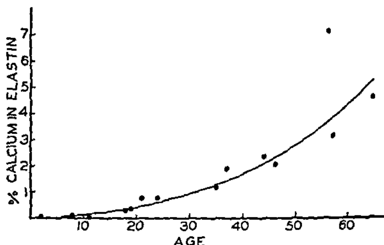


Fig 2 Age variations in calcium content of elastin in the media of human aortas grossly free of atheromata Data calculated on a dried defatted basis

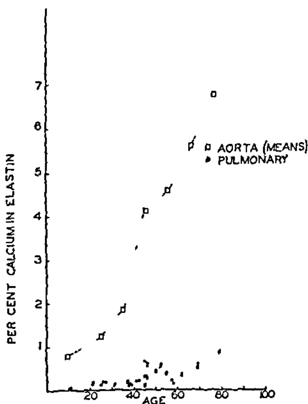


Fig 3 Increase of calcium content of pulmonary arterial elastin with age compared with values for the aorta Note the marked difference in calcification with age in these vessels From Lansing *et al* (5)

First elastin is arbitrarily defined It is called a scleroprotein and as such is one of the most insoluble materials in the body The methods that we use simply depend upon removing materials which are soluble and calling that which is left over elastin Most laboratories use diverse methods of preparation of elastin which involve leaving behind residual and variable amounts of collagen and other tissues In doing this some come up with apparent carbohydrate moieties while we come up with no carbohydrate The carbohydrate is probably derived from contaminating collagen When one applies enzymatic procedures to elastin the results depend on the degree of homogeneity of the substrate This is one of the headaches in this field

Another problem that has come up repeatedly is a lack of well defined ultrastructure in the elastic fiber which has hampered elastic tissue research for many years I might say that we do not have in elastin the beautiful fine structure that we have in collagen Collagen exhibits a neat transverse periodicity of 640 A while elastic tissue is almost entirely amorphous It is sometimes very difficult to recognize in the electron microscope

We still are handicapped by the unclear source of elastase My laboratory thinks that it is a product of islet tissue There is just as good evidence perhaps better to show that it is not a product of islet but rather of acinar tissue We have been confirmed and we have not been confirmed It would be a great help if this issue could be clarified

We have no clear notion as to the function of elastase This is still highly controversial and a subject of study in at least a few laboratories There is still some reason to believe that elastase acts systemically at least in the rabbit, as we have used it, to influence fatty livers and the course of atheromatosis Elastase is an enzyme and yet, it appears to be acting systemically To make it still more complex the best results we have gotten so far until recently have been with oral administration of elastase and a protein should not be effective in this way

Lastly we now have good reason to believe that there is a material proelastase which can be separated from pancreas Trypsin acts on proelastase which is inert to yield active elastase We now must determine the relationship between proelastase and elastase Is this the active systemic component or is it not? Obviously it will be at least a few years before these questions will be answered

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Discussion

Chairman Hartroft Although it is interesting that atherosclerosis was apparently present in centuries past and although it is not restricted to humans I think we should keep clearly in mind the complications of atherosclerosis particularly myocardial infarction and other disturbances that appear exclusively in human disease as we know it Reports of spontaneous myocardial infarction in animals are very rare

Secondly although it may have been present in the Egyptians I think the evidence of this decade or this century shows clearly the complications of this disease are increasing

Finally there is no direct relation of at least the early stages of atheroma to the incidence of its implications The St Louisean Negro has as much atheroma before age 20 or 30 as the white has and actually has more yet myocardial infarction is less common in the St Louisean Negro Similarly data from New Orleans are very convincing Pullman shows identical results comparing Guatemalan Indians and New Orleans whites Atheroma is more common in its early stages in the Guatemalans than in the New Orleans whites yet myocardial infarction is almost unknown in Guatemalans

Dr Lansing when you showed the medial change and then the intimal change in St Louisean whites did you believe the medial changes came sooner than the intimal?

Dr Lansing That is what I hoped you would infer

Chairman Hartroft Do you believe then that atheroma is initially a medial change?

Dr Lansing No I believe that damage to the arterial wall predisposes the vessel to changes in the intima I believe that intimal fibrosis comes before lipid infiltration I believe that the change in the arterial wall is a predisposing factor

Chairman Hartroft You mean the media?

Dr Lansing At the moment I am referring to two lesions—luetic aortitis and elastic tissue breakdown involving the media

Chairman Hartroft Then the media does come first in your hypothesis?

Dr Lansing Yes Damage to the media of the arterial wall as far as I can determine from the human data precedes intimal involvement

Chairman Hartroft Writing on current concepts of the pathogenesis of atheroma I said I thought everybody would agree it started out with the intima with one exception—Dr Lansing

Dr Lansing You are quite right

Dr Moon Dr Hartroft I have the impression that Dr Lansing considers the

internal elastic membrane as part of the media in other words he is not considering the internal elastic membrane as a separate layer

DR LANSING I referred to the elastica interna as a component of the media

DR ALLEN I think you have made a very good point Dr Hartroft about the difference between atheromatosis and atheromatosis with complications The most important thing about atheromatosis clinically is that it interferes with the major function of the artery—transportation of blood Dr Lansing have you ever seen acute myocardial infarction cerebral thrombosis intermittent claudication or gangrene occur spontaneously in chickens pigs bears otters skunks or sheep?

Secondly you have shown atheromatosis occurs in the veins lymph vessels and pulmonary arteries As a clinician I recognize no syndrome of the complications of atheromatosis in these areas

Thirdly you said atheromatosis is usually associated with hypercholesteremia Using 250 mg percent as normal for an adult about one half of all patients with complications of atheromatosis have normal cholesterol values

Finally you stated atheromatosis was a function of age or closely related to age Maybe the lesion of atheromatosis is but the complications of atheromatosis are related only in a very gross way to age for we have observed serious complications of coronary atherosclerosis in children and we have seen nonagenarians who have no such sequelae

And then I make a plea for differentiating between the lesion which is called atheromatosis and the lesion of atheromatosis which interferes with the transportation of blood

DR LANSING I thought that was differentiated when the symposium was set up We are considering connective tissue and atherosclerosis, not connective tissue and complications of atherosclerosis I think that provides the answer

I tried to describe atheromatosis or atherosclerosis in animals because it occurs in animals as well as in human beings The fact that myocardial infarction does not occur in the animal or in veins or in lymph vessels is immaterial at this point We are trying to gain an understanding of the genesis and management of the changes that occur in these vessels

The mechanisms of thrombosis and of myocardial infarction are entirely separate problems I do not think these comments are germane to our symposium

CHAIRMAN HARTROFT What both Dr Allen and I were a little overly anxious to bring out was the statement you made which was perfectly correct that atherosclerosis the lesion may occur in all these different situations and in different species Nevertheless we would not want anybody to think the complications occurred as well.

DR GITLIN Can somebody answer the question as to whether or not the complications really do not occur in animals?

DR ADLERSBERG How do you know they don t?

DR GITLIN Many of these animals are slaughtered long before they reach the ripe old age of human beings

CHAIRMAN HARTROFT The veterinary school at Ithaca published on the development of atherosclerosis in their prize bulls and horses These animals are now kept alive a long time until they are absolutely infertile As a result these valuable animals have been carefully followed well past what would be comparable to the myocardial infarction age in man Ulceration and calcification of various vascular lesions which some of us regard as a complication also as well as thrombosis have not been found

DR. LANSING Dr Hartroft you referred to the fact that the complications of atherosclerosis lag behind in the Negro whereas the lesions themselves do not I think that depends entirely upon the vessels to which you refer in fact I am not entirely sure of the observation

Some years ago in St Louis when Dr Blache compared white and Negro populations there was no distinction as far as age incidence of atherosclerosis was concerned The atherosclerotic changes in the coronary in the Negro trailed by a decade but in the cerebral and renal arteries they were ahead by a decade One has to be very specific as to the vessel in the Negro to which one is referring

CHAIRMAN HARTROFT Strong and Pullman reported the so-called lipid streak or the pearly plaque is more advanced in the Negro in the early stage

DR. BOUCEK It is just the lipid streak Recently they found no correlation between the lipid streaking and the development of the pearly or fibrous plaque

DR MOON Myocardial infarction has been described in the dog and in the horse

CHAIRMAN HARTROFT I think it is a very low incidence

DR VITALE With regard to complications of this disease in animals I think it depends upon the age of the animal Dr Gottlieb has seen in very young animals fed just cholesterol and cholic acid with no added thiouracil extravasation of blood into the myocardium—consistent with an early myocardial infarction It is true however that it remains to be proved that this early lesion will go on to infarction

DR GROSS With regard to the connective tissue elements one interesting thing about elastin is that it supposedly does not regenerate very readily Emerson and Nathanson observed in cancer of the breast treated with estrogens there is an extremely rapid proliferation of great masses of material which as far as the histologist can say is elastin I wonder whether Dr Lansing has any comments on the mechanism of the formation of elastin

DR LANSING No I do not Usually it is considered to be refractory insofar as new growth is concerned The postpartum uterine artery is an exception Beyond that I am not aware of any direct evidence for active formation of elastic elements in normal adult tissues

DR GLIMCHER In the material you presented with reference to the increase in calcium content with age I am interested to know if at any stage the calcium was merely bound by itself or was the calcium always in the form of crystalline calcium phosphate?

DR LANSING We are satisfied that it is an apatite like crystal according to the calcium carbonate-phosphate ratios The calcium salt can be dissolved entirely using weak acid and then by raising the pH one can recombine as much as 15% of the original calcium to elastin However in elastin the structure of the calcium salt appears to be an apatite crystal

DR BOUCEK It is difficult to appraise the physical properties of elastin in tissues such as an artery Does the reaction of elastin with 88% formic acid assist in this matter?

DR. LANSING I honestly cannot say I know that the apparent elasticity that one gets in elastin preparations after formic acid preparation and after sodium hydroxide preparation are very different indeed But we have not made an organized study

DR BOUCEK Haas thought there was a rough correlation between the lack of swelling with formic acid and age

DR LANSING Yes He and I have discussed this point and we are back again

to the definition of elastin and the method of preparation It needs further elaboration

There is also the question as to whether there is loss of elastic tissue in vessels as the function of age Using methods that we have modified from Lowry the constancy of elastin as a function of age and the amount of extractable elastin in vessels is clear It is only less elastic granular and fragmented

DR WINTER I would like to know what elastase by mouth does? Does it act like an enzyme? Is it a protein material extracted from the pancreas?

DR LANSING I do not know what it does but if rabbits are given Purina chow and 0 3% cholesterol in cotton seed oil daily and if this chow is supplemented with that amount of elastase that can be extracted from 5 gm of pancreas almost total inhibition of the fatty livers and marked inhibition of atheromatosis results Three independent series gave us these results in 1954 They are currently being repeated with a much more refined preparation of elastase using oral preparations and intraperitoneal injections The results now are not far from the original observations of 1954

DR. MOON What are the conditions for converting proelastase to elastase?

DR LANSING The method that Grant and Robins and we have used is trypsin activation

Chemistry of Ground Substances*

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The field I am going to discuss has been reviewed in the last years in several articles For this reason I will only sketch briefly the present status of our knowledge of the chemistry of the ground substances The concept of ground substances of connective tissue originated from histological investigations The proteinaceous extracellular material which under the light microscope appeared outside the fibrous elements was named "Grundsubstanz It originally was believed to represent an amorphous gel from which the fibrous elements formed by some process of crystallization This concept nowadays is at best only partially correct and the term "ground substance" cannot be defined chemically except in operational terms In general one may define the ground substances as the material soluble in aqueous salt solutions under mild alkaline conditions It has to be stressed here that by this treatment substances originating in the formed elements both fibrous and cellular are included Furthermore some of the substances originating in the extrafibrillar spaces are altered and not obtained in their native states In fact some of the acid mucopolysaccharides which are generally considered to be typical constituents of the ground substances have been obtained as their native protein complexes only from a few tissues such as the protein complex or complexes of hyaluronic acid from synovial fluid or the protein complex of chondroitin sulfate from hyaline cartilage

A discussion of the chemistry of the ground substances usually deals only with the acid mucopolysaccharides which have been isolated from various sources after enzymatic digestion of the proteins and rigorous purification Plasma proteins which form a major part of the water soluble extractives of connective tissue are of course eliminated by these methods Other protein bound carbohydrates of connective tissues have been described which may be components of the ground substances but most probably belong to the fibrous elements At present they are very poorly defined and I will omit them from this discussion

*Supported in part by U S Public Health Grant

Acid mucopolysaccharides are present in all connective tissues although in greatly different amounts varying from approximately 20% of the dry weight in hyaline cartilage to less than 0 5% in dense adult connective tissue As a rule the content is higher in young connective tissue concomitant with their higher water content

We have attempted in our laboratory to investigate and quantitate the distribution of the mucopolysaccharides in various sites of connective tissues In the course of this work it became obvious (*1*) that the number of distinct mucopolysaccharides was far greater than was known before and (*2*) that the types and quantities of mucopolysaccharides differed in various sites of normal connective tissues and that these patterns differ in embryonic tissue and to some extent in abnormal tissue However changes in the mucopolysaccharide pattern in disease thus far have been investigated only to a very limited extent

TABLE I
ACID MUCOPOLYSACCHARIDES OF CONNECTIVE TISSUE

I Nonsulfated mucopolysaccharides
1 Hyaluronic acid
2 Chondroitin
II Sulfated mucopolysaccharides
3 Chondroitin sulfate A
4 Chondroitin sulfate B
5 Chondroitin sulfate C
6 Keratosulfate
7 Heparitin sulfate

Table I contains a list of the acid mucopolysaccharides which have been identified thus far In this table are listed two nonsulfated acid mucopolysaccharides hyaluronic acid and chondroitin The structure of hyaluronic acid has been established by a combination of chemical and enzymatic methods It is a β 1 4 linked unbranched polymer of a disaccharide *N* acetylhyalobiuronic acid or β D glucosylpyruronic acid 3 *O N* acetylglucosamine (1) The structure of hyaluronic acid is represented by Fig 1 Chondroitin the second polysaccharide listed in Table I resembles hyaluronic acid in properties and structure However we have never succeeded in obtaining it completely free of ester sulfate The best preparations contain about 2% sulfate while hyaluronic acid of course is free of sulfate Chondroitin has been found only in cornea Chemically *it is the N acetylgalactosamine isomer of hyaluronic* acid Its structure is based on the isolation in good yield of crystalline chondrosine the repeating disaccharide identical in its infrared spec

trum with chondrosine isolated from chondroitin sulfate A or C (see below) The enzymatic hydrolysis by testicular and bacterial hyaluronidases quantitatively gives the galactosamine isomers of the di and oligosaccharides obtained by these enzymes from hyaluronic acid

We now turn to the sulfated mucopolysaccharides The most widely

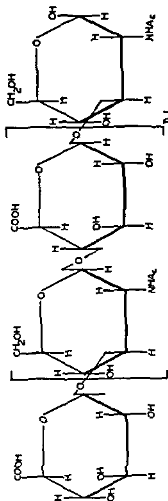


Fig 1 Structure of hyaluronic acid

distributed and quantitatively the most important are the chondroitin sulfates which we have designated as ChS A B and C Actually there are apparently in the mammalian organism still more polysaccharides composed of equimolar quantities of hexuronic acid N acetylgalacto

samine and varying equivalents of sulfate A and C have identical repeating units namely chondrosine isolated in crystalline form in good yields from both The structure of chondrosine has been established as β D glucosylpyruronic acid 3-O D-galactosamine i e it is the 4 epimer of hyalobiuronic acid (2) The *N* acetylated disaccharide as in hyaluronic acid is polymerized by β 1 4 hexosaminidic bonds to polymers of a molecular weight of approximately 40–50 000 (2) The only difference between A and C appears to be the position of the sulfate ester group which in A is in the 4 position of the galactosamine and in C in the 6-position (see Fig 2) This conclusion is based on the similarity of the

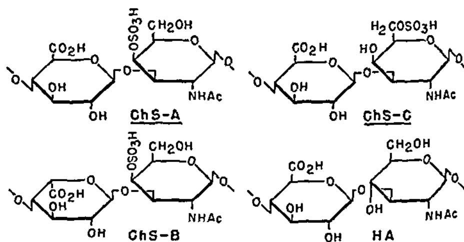


Fig 2 Structure of chondroitin sulfate A B and C compared to that of hyaluronic acid (HA)

infrared spectrum of ChS A with that of B The latter has been shown recently by Stoffyn and Jeanloz by chemical means to have the sulfate in 4 position We have shown previously that the sulfate groups of both A and C were in the hexosamine moiety which leaves for ChS C only the 6 position for esterification

Chondroitin sulfate A and C occur in cartilage and bone as well as in most soft connective tissue ChS C appears to be typical for young and more primitive connective tissue (shark cartilage electric eel, fibroblasts in tissue culture)

The structure of chondroitin sulfate B is less well established than is that of A and C Its salts are less soluble in aqueous alcohol than those of the latter A comparison of carbazole and orcinol reactions for hexuronic acid lead to the suspicion that the hexuronic acid in ChS B

was largely iduronic acid, the 5-epimer of glucuronic acid (Fig 3) The presence of iduronic acid was confirmed by paper chromatography (8) Recently Stoffyn and Jeanloz isolated from ChS B crystalline derivatives of L iduronic acid It was mentioned above that these authors also established the position of the sulfate group on carbon-4 of the galactosamine and the 3 position of the uronidic group (4) The hexosaminidic group is in all probability linked to the 4 position as in hyaluronic acid in ChS A and in C This conclusion is based on the formation of a Δ-4 5

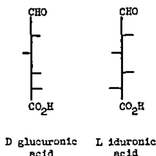


Fig 3 Structure of D glucuronic acid L iduronic acid in Fischer projection

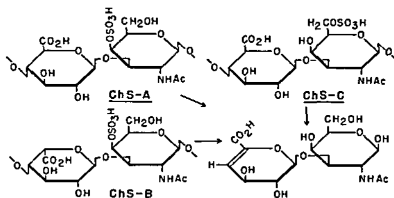


Fig 4 Formation of the identical Δ 4 5 unsaturated de ulfated disaccharide from ChS A B and C

unsaturated uronide by extracts of a flavobacterium adapted to ChS B which simultaneously desulfate the carbohydrate (5) As could be expected this unsaturated disaccharide formed from B is identical with that formed from desulfated A or C (Fig 4) Chondroitin sulfate B occurs normally in skin ligamentum nuchae aorta tendon and heart

valves It is absent from cornea cartilage and bone Embryonic skin contains ChS B only in very small concentration (6) All chondroitin sulfate fractions which have been prepared by a variety of methods and from a variety of sources in our laboratory or which we have obtained from other workers contain both iduronic and glucuronic acid in ratios varying between 4 to 1 and 1 to 1 They all are resistant to testicular hyaluronidase and appear to be hybrid molecules (7)

The next sulfated mucopolysaccharide is keratosulfate Its structure is unknown it is nondialyzable and composed of *N* acetylglucosamine galactose and sulfate in equimolar proportions Two tissues contain keratosulfate in relatively large quantities cornea (8) and nucleus pulposus (9) In both it constitutes about one half of the total mucopolysaccharide The other half in cornea is ChS A and chondroitin in nucleus pulposus it is ChS C It was further isolated as a minor constituent from the lower ends of the long bones of calf (10) A pattern similar to that of nucleus pulposus was recently encountered in the rib cartilage of two cases of Marfan s syndrome Both were young men in their early 20s who had died of dissecting aneurysm of the aorta The aorta of one of these cases which also has been studied likewise yielded keratosulfate in addition to the complex mixture of mucopolysaccharides *isolated from normal bovine aorta On the basis of this finding the rib* cartilage of a control case was investigated that of a 23 year old man who had died suddenly after an operation for the removal of a brain tumor The presumably normal cartilage of this case yielded also kerato*sulfate beside chondroitin sulfate however in a ratio of only* 3 to 1 Since the finding of keratosulfate in cartilage was rather unexpected a renewed investigation of the composition of cartilage is imperative

The last member of the sulfated mucopolysaccharides listed in Table I heparitin sulfate is chemically closely related to heparin Both contain D glucosamine and D-glucuronic acid they are dextro rotatory resistant to testicular and bacterial hyaluronidase and give *high carbazole and low orcinol values Extracts prepared by* Dr Korn of the National Heart Institute from a flavobacterium adapted to either heparin or heparitin sulfate will hydrolyze equally well both polysaccharides (11) and the pattern of the hydrolysis products from both are very similar While heparin contains between 2 5 and 3 sulfate groups per repeating disaccharide one of which as *N* sulfate and is free of acetyl heparitin sulfate has maximally one sulfate group per disaccharide unit approximately one half as *N* sulfate and one half as *O* sulfate The rest of the amino groups are *N* acetylated (11) Heparitin sulfate furthermore is devoid of anticoagulant properties and it has a low activity *as a lipemia clearing factor The structures of*

heparin and heparitin sulfate are unknown Heparitin sulfate slowly passes dialysis tubing It apparently is identical with the heparin mono sulfate isolated from beef lung by Jorpes and Gardell in 1948 (12) In our laboratory it was isolated first from livers with secondary amyloido sis and later from bovine and human aorta This mucopolysaccharide aroused considerable interest when it was encountered in the livers and some other organs and in the urine of children with Hurler s syndrome or gargoylism (13) The urine of most of these patients contains chondroitin sulfate B beside heparitin sulfate the former as a rule in larger quantities while the organs in this disease apparently store and contain as intracellular inclusions mainly if not exclusively heparitin sulfate However we recently have isolated from two brains of children with gargoylism chondroitin sulfate B a polysaccharide not found in

TABLE II
ANALYSIS OF HEPARITIN FRACTIONS AND OF HEPARIN

Source	Uronic acid (%)			Hexosamine (%)	$[\alpha]_D$	Sulfate (%)	Acetyl (%)
	carbazole	orcinol	CO_2				
Liver amyloid	35	24	34	29	+39	16	5 5
Aorta	36	20		29	+58	10	
Urine Hurler s syndrome	39	19		24	+60	6	
Liver Hurler s syndrome	47	17	28	28	+69	11	4 1
Heparin side fraction[b]	37	19		28	+54	16	4 1
Heparin side fraction[b]	36	20	25	28	+60	10	4 0
Heparin (Organon 126 units/mg)	40	9	21 8	21	+52	33	0

Analytical methods have been described previously
[b] We thank the Upjohn Company for this material

normal brain and as a second polysaccharide heparitin sulfate in one case in somewhat lower quantity than B while the other brain con tained a large quantity of ChS B and a small quantity of heparitin sul fate In Table II (5) analytical data of heparitin sulfate of different sources are listed together with a typical heparin fraction It can be seen that the analytical values of the heparitin sulfate fraction from different sources are very similar as are their infrared spectra Whether the observed differences in analytical values are due to contamination or whether heparitin sulfate represents a family of compounds similar to the chondroitin sulfates cannot be decided at present It might be mentioned here that a heparin monosulfate prepared from heparin by chemical desulfation according to Kantor and Schubert is not hy drolyzed by the heparin adapted enzyme Heparin monosulfate thus

prepared has free amino groups in contrast to heparitin sulfate in which half the NH groups are acetylated half sulfated

As to the biological functions of the acid mucopolysaccharides it may be assumed that the nonsulfated mucopolysaccharides bind water and form a major component of the amorphous gel in interstitial spaces Hyaluronic acid or its protein complexes further acts as a lubricant between articular surfaces and tendon sheaths and forms a barrier perhaps oriented in the fibrous meshwork of the chamber angle of the eye The latter function has been most forcefully demonstrated by the experiments of Barányi on the flow of the aqueous humor (14) The relatively large concentration of hyaluronic acid in ligamentum nuchae and in normal aorta and its apparent absence in immobile tissue such as the sclera points to its role as a lubricant between elastic and their surrounding collagen bundles It might be of interest to point out here that in aortas of old persons (median age 64 years) the hyaluronic acid fraction was markedly decreased if not absent

The biological function of the chondroitin sulfates and probably of keratosulfate (as their protein complexes) is suggested by the action of papain on the rabbit ear by which the protein complex of chondroitin sulfate is removed leading to the collapse of the ear From these experiments and from electron microscopy it can be concluded that the protein complexes of chondroitin sulfate form the interfibrillar bridges of the collagen bundles and act as the sizing of this fabric It has been pointed out previously that chondroitin sulfate A and especially C are associated with the fine immature and more soluble and B with the coarse mature collagen fibers The role of keratosulfate and its localization in connective tissue is obscure Still less is known about the location and function of heparitin sulfate Its molecular weight is considerably below that of the chondroitin sulfates While it is undoubtedly chemically very closely related to heparin its biological relationship to heparin is unknown For example children with gargoylism and with excretion of large quantities of heparitin sulfate in their urine showed no abnormalities of blood coagulation In the literature on the histology of tissues from cases of gargoylism no abnormal number of mast cells has been reported Compared to heparin its action as a lipemia clearing factor is weak while that of ChS B is comparable to heparin (private communication from Dr J Seifter)

On the other hand profound vascular changes occur in gargoylism and coronary occlusion is frequently the cause of death in young children with gargoylism In preliminary experiments by Mr Morris and Dr Godman it was shown that macrophages in tissue culture take up added heparitin sulfate from the culture medium and store it in the

protoplasm in the form of large discrete metachromatic granules similar in size to those of eosinophiles It remains to be seen whether the chronic administration of this polysaccharide will lead to the vascular and cellular changes typical for the disease

In summary this paper was intended to review the chemistry of the ground substance or more specifically of the acid mucopolysaccharides of the so called ground substance The complexity of these fractions has been emphasized and the attempts have been described to classify and identify by chemical physical and enzymatic methods the isolated polymers Interpretation of the biological role and the integration of the histological and pathological connective tissue structures with the observed mucopolysaccharide patterns has been attempted although such attempts have to be at present only very tentative

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Discussion

Dr Glimcher In the staining techniques of tissue for the whole group of mucopolysaccharides a number of statements have been made relative to the cor relation between staining techniques and the degree of aggregation of the muco polysaccharides Would you comment on that?

Dr Meyer This goes back to the degree of metachromatic staining which is given most prominently by high polymers and not by low in fact this is most clearly demonstrated *in vitro* in hyaluronic acid I did not say anything about molecular weight but there are hyaluronic acids even without being protein com plexes in which the hyaluronic acid is of a very high molecular weight These are strongly metachromatic If they are split with hyaluronidase they lose the meta chromasia completely

Chairman Hartroft Have you any comments about Hurlers syndrome Dr Angevine?

Dr Angevine No I do not

Dr Page Do I understand that Hurler s syndrome is one of the amyloid dis eases?

Dr Meyer Yes this is a connective tissue storage disease A very high in cidence of children with Hurler s syndrome develop narrowing of the arteries throughout the body and die of coronary disease There is no fat deposition at least not in the artery Of course this does not explain the disease rather some of the symptoms The disease is usually a genetically determined defect with the appearance of two different polysaccharides which chemically are unrelated to each other so that the defect must lie somewhere else

Dr Moon Is there any correlation between age and the degree of polymeriza tion of the mucopolysaccharides in general?

Dr Meyer No Drs Isidore Gersh and Catchpole erroneously made the state ment on the basis of the appearance of the metachromatic substances in connective tissue in scurvy in malignancies etc in the region of advancing tumors and the simultaneous appearance of hexosamine containing compounds in the blood

These hexosamine-containing compounds have nothing to do with the substances I discussed Whether they come from the connective tissue is not known I would doubt it

Dr von Kaulla Is anything known about these compounds in the way of antihyaluronidase? Heparin is an antihyaluronidase

Dr Meyer They are not digested they are not efficient antihyaluronidases They are made like all compounds that are more strongly polar than hyaluronic acid is or than the carboxyl group in hyaluronic acid is They do interfere to some extent competitively but this has nothing to do with the so-called serum inhibitor of hyaluronidase of which so much has been made with so little to show for it

Chairman Hartroft Would you classify this with Niemann Pick s disease?

Dr Angevine No it is not that type of storage disease I think I have seen only one other than Dr Meyer s This is more closely related to Marfan s syndrome and is more a developmental defect of the mesenchyme

Dr Meyer Yes except these children did not start out with defects in con trast to the chondrodystrophy where it is really a dystrophy of the anlage These develop slowly

This has something to do with the organization of the collagen into the struc tural units in contrast to the collagenous molecule The specificity of the fibroblasts must have some role in producing these various products in different sites differ ent concentration and even different types If you find in bone for example or in cartilage chondroitin sulfate B which normally is not produced there this in our opinion would change the organization of the bone as an organ

Dr Ratnoff I am fascinated by the chemistry but what is all the muco polysaccharide doing there? What is its function?

Dr Meyer Their function is deduced from these phenomena You can show in vitro that hyaluronic acid is an excellent lubricant Dr Ogston has deduced from its structure and its physical properties that it would be an excellent lubricant and apparently it is because we walk on a layer of hyaluronic acid Our whole weight is held up by it and if it is missing cartilage disappears from the joint As you know hyaluronic acid disappears in osteoarteritis

When hyaluronic acid is found in excessive amounts in skin under certain con

ditions it produces edema It holds water binding water in various interstitial spaces I am leaving out heparitin sulfate of which we are ignorant Keratosulfate may also be a component of the ground substance but we do not know We do not know why the main polysaccharide in the cornea (over half the total) is kerato sulfate In nucleus pulposus there is a 50-50 mixture half chondroitin sulfate C and half keratosulfate The different sulfated polysaccharides occur or are corre lated with different appearing collagen fibers as far as the size or width of the fibers is concerned

We believe they are part of the finer interfibrillar network shown by electron microscopy on teased preparations of very many tissues In addition the polar groups must have some function or are concerned with binding of cations by cartilage for example

DR. WHITE Dr Meyer referred to what I think has already been demonstrated experimentally and will be an extraordinarily important function physiologically of the mucopolysaccharides namely their capacity to act as ion exchange resins in tissues T F Dougherty and R D Higginbotham (*Proc Soc Exptl Biol Med* 96 466 1957) showed these substances will bind basic materials e g histamine pro tamine and polymyxin B Also there is evidence provided by Dr Frank Engel which suggests some of the extra adrenal effects of ACTH (the action of ACTH in the adrenalectomized animal) might be explained by the fact that ACTH is a basic peptide bound by tissues and can then be released Not only do the mucopolysac charides bind basic substances but the latter can be displaced by more basic com pounds from these binding sites

DR MEYER This is yet another role beautifully illustrated by Dr L Thomas on the rabbit ear When he injected papain the ears flopped down The collagen fibers are flabby and this makes it stiff He has shown that papain disappears from blood in very short time apparently and is bound to connective tissue It does not act on the polysaccharide but on the protein complex When the protein complex is removed the ear is wilted When it is replaced the erect position of the ear is resumed

Properties of Fibroblasts

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Department of Research Howard Hughes Medical Institute Miami Florida

Fibroblasts the preponderant cell type found in loose connective tissues are widely distributed cells of mesenchymal origin The name fibroblast refers to the ability of the cell to elaborate collagen fibers but there is evidence that many components of the ground substance are also formed by this cell While the fibroblast is of importance in the maintenance of connective tissue its major activity is exhibited in the formation of new connective tissue components during embryogenesis and growth and during regeneration and repair

When injury occurs in the body a universal response which presages the process of repair is a mobilization of fibroblasts Wounding of the skin results in the appearance of fibroblasts within a matter of hours The fracture of a bone causes the development of large numbers of fibroblasts which bridge the bony fragments prior to the formation of a callus Injury resulting from hemodynamic trauma such as a jet lesion distal to a coarctation or at vascular angulation causes an increased concentration of fibroblasts in the intima at the damaged site The implantation of a relatively inert structure such as a plastic sponge into which the fibroblasts can migrate is an easily reproduced injury and provides a means to biopsy normal appearing tissue Such a technique has been employed in our laboratories (2) and has permitted a study of the effects of species sex of the animal and tissue age upon biochemical properties of connective tissue Characteristics of the activities of fibroblasts obtained by sponge implantation form the substance of this report

After the sponge has been implanted its interstices rapidly become filled by a homogenous eosin staining material and by the third day the periphery becomes surrounded by a capsule of fibroblasts Five days postimplantation the eosin staining material coalesces into argyrophilic strands which serve as thoroughfares for the invading fibroblasts Capillaries develop early in the tissue particularly around the outer zones of the sponge Within 5 to 6 days chemically and tinctorially detectable collagen is noted Metachromasia is observed only in the very young tissue The coalesced strands which appeared early are removed and

replaced by collagen tissue Deoxyribonucleic acid determinations (Fig 1) indicate that the cell population remains stable after the 12th to 14th day (2 27) It appears as though the body allotted a given number of fibroblasts in response to the injury of sponge implantation and that the number was determined by the extent of the injury

I FORMATION OF COLLAGEN

The collagen molecule is formed within the fibroblast (10), possibly on or within the endoplasmic reticulum as suggested by Porter (21) and then extruded into the surrounding extracellular space The presence of hydroxyproline in concentrations of 13–14% by weight (14) is unique to collagen Another hydroxy amino acid hydroxylysine has been found only in this protein The "parent" amino acids proline and lysine are hydroxylated after incorporation into the polypeptide strand (23 25) Hydroxyproline occupies a critical position in the formation of collagen and is one of the principal amino acids involved in the stabilization of collagen through the formation of hydrogen bonds involving the hydroxyl group (7) Lysine and hydroxylysine may be involved through their epsilon amino group in the cross linking of peptide chains (24)

TABLE I
In Vitro HYDROXYLATION OF C^{14}-LABELED PROLINE BY CELLULAR PARTICLES USING 12 DAY OLD MALE RAT CONNECTIVE TISSUE

Cellular particle added	μg C^{14}-labeled hydroxyproline × 10^{-4} per flask*
Nuclei mitochondria microsomes	3 0
Mitochondria microsomes	5 5
Microsomes	1 5
Nuclei	2 0
Mitochondria	2 2
None	4 2

* Flask contained collagen fiber 105 000 × g supernatant, ATP + phosphocreatine C^{14}-L-proline plus added particle

The process of hydroxylation of the amino acids proline and lysine is one of the principal steps in collagen biosynthesis To study acellular hydroxylation the cell particles of 12 day old fibroblasts were isolated by differential centrifugation (Fig 2) The cell fractions were incubated aerobically with L proline C^{14} in a buffered solution containing an energy system of adenosine triphosphate and phosphocreatine The contents of the incubation flasks were dialyzed and hydrolyzed Hydroxyproline and proline were separated by column chromatography (22)

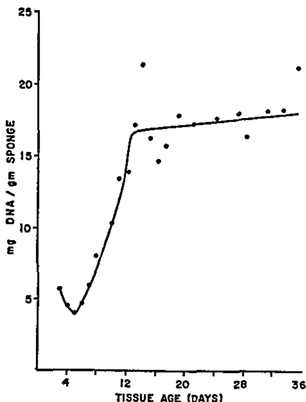


FIG 1 Deoxyribonucleic acid in implanted sponge

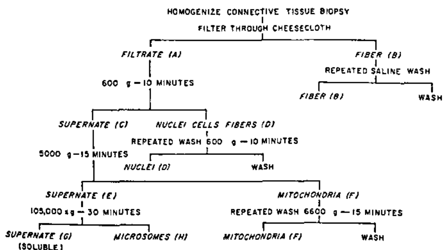


FIG 2 Preparation of fibroblasts

and quantitated (14 26) C^{14} labeled hydroxyproline was formed in the presence of the 105 000 × g supernatant (Table I) and incorporated into a peptide chain Hydroxylation in the supernatant fraction was enhanced by the addition of microsomes and mitochondria and depressed by addition of nuclear material Boiling the supernatant caused a marked reduction of hydroxylation

Thus the process of hydroxylation so critical to collagen formation was demonstrated to occur in the absence of the intact fibroblast The enzymatic nature of the hydroxylation was suggested by the heat lability of the system

1 *Formation of the Ground Substance*

While a large body of information is available relevant to collagen little is known about the equally important and probably more metabolically dynamic ground substance Since the early 1930s Karl Meyer has pioneered studies on the polysaccharides of connective tissue and has elucidated the chemical structure of a number of these substances The principal components of the mucopolysaccharides are the amino sugars and uronic acids The amino sugars glucosamine and galactosamine are thought to arise from the fibroblasts The separation of these amino sugars by column chromatography (6) and their quantitation (1) revealed that only glucosamine was present in the sponge connective tissue at the time that collagen was detected (Table II) (17) During the initial 20 days of tissue age the largest percentage of the

TABLE II
HEXOSAMINE IN CONNECTIVE TISSUE OF MALE RAT

	Saline-Soluble		HAc-Soluble	Residue	
Tissue age in days	Glucosamine (mg /gm sponge implant)	Galactosamine (mg /gm sponge implant)	Total (mg /gm sponge implant)*	Glucosamine (mg /gm sponge implant)*	Galactosamine (mg /gm sponge implant)*
6	2 79	0 0	0 18	0 93	0 0
7	3 23	0 0	0 23	1 28	0 0
9	2 44	0 0	0 25	0 86	0 24
11	2 22	0 0	0 24	1 22	0 35
13	2 51	0 0	0 21	1 19	0 54
15	2 15	0 68	0 31	1 28	0 86
20	2 24	—	0 30	1 51	0 77
27	2 27	0 74	0 61	1 27	0 84
34	1 80	—	0 51	1 13	0 73

Average values for two animals at each tissue age

TABLE III
HAc-Soluble Fraction of Fibrous Protein in Male Rat Connective Tissue

Tissue Age in Days	Per cent of total collagen*	Per cent of total hexosamine	Hydroxyproline gm /100 gm protein
6	22 9	4 6	1 1
7	22 4	4 8	2 6
9	—	6 6	2 2
11	8 2	6 0	2 9
13	—	4 7	3 6
15	11 0	5 9	5 1
20	17 [illegible]	6 2	8 2
27	22 1	10 6	7 0
34	27 6	12 2	8 3

* Average values for two animals at each tissue age

total glucosamine was found in the 15 000 × *g* supernatant of the saline homogenate of sponge connective tissue Extraction of the residue with 0 5 *M* acetic acid (HAc) solubilized a portion of the hexosamine and collagen (Table III) In the 6–9 day old tissue a large amount of protein other than collagen was also solubilized by acetic acid resulting in a low percentage of hydroxyproline in the acid extract (1–2%) The percentage of hydroxyproline in the HAc extract rose to 7–8% by the 20th day of tissue development All of the hexosamine in the young tissue (6–9 days) was glucosamine probably existing either as hyaluronic acid or as glycoprotein

Galactosamine appeared in the saline insoluble fraction by the 9th day of tissue development A temporal relationship between the glucosamine and galactosamine existed in the saline soluble and insoluble fractions in that when galactosamine appeared the concentration of glucosamine decreased Galactosamine was found in the saline soluble fraction at the 15th day of tissue age

The appearance of galactosamine in the tissue coincided with an alteration in the solubility of collagen in 0 5 *M* HAc (Tables II and III) When only glucosamine was present in the saline insoluble residue approximately 25% of the total collagen was solubilized by acetic acid With the appearance of galactosamine the acid soluble collagen was reduced to approximately 10% Galactosamine has been reported to exist in combination with collagen in the form of chondroitin sulfate (8 9) The observed relationship between the galactosamine content and collagen solubility is further suggestive evidence of the stabilizing influence of chondroitin sulfates upon collagen

2 Synthesis of Cholesterol

Early in the studies of biopsy connective tissue it became apparent that high concentrations of lipids were present (4 15) Only the brain surpassed the biopsy tissue in concentration of cholesterol and no other mammalian tissue had as high a concentration of the esters of cholesterol A species difference in the lipid characteristics of the sponge tissue was noted The cholesterol concentration was highest in the tissue obtained from man and from chicken (3)

To determine the ability of the fibroblasts to synthesize cholesterol slices of the sponge connective tissue were aerobically incubated in a Krebs Ringer bicarbonate solution containing 0 5 ml of C^{14} carboxyl labeled sodium acetate (0 82 mg of sodium acetate) for 11 hours at 37 5°C After saponification the cholesterol was extracted with petroleum ether and carrier cholesterol added The digitonide was precipitated filtered placed on a planchet and its radioactivity counted (16) The cells of the sponge tissue synthesized a digitonin precipitable substance from the labeled 2-carbon fragment This synthetic ability of the fibroblast was destroyed by boiling the tissue slices The newly formed labeled digitonin precipitable substance was present in the saline soluble fraction containing the protein of the ground substance in the fibroblasts separated from the other tissue components by 0 25 *M* sucrose and in the collagen fraction The major portion of the labeled material was found with the cells and in the ground substance

II Enzymes of Connective Tissue

The heat lability of the systems used in the studies of the acellular hydroxylation of labeled proline and of cholesterol synthesis was suggestive of enzymatic processes Little information is available on the enzyme systems of connective tissue Woessner in our laboratories has surveyed the enzymes of developing connective tissue in the sponge implant (27) The sponge enzymes were prepared by filtration and centrifugation of the homogenate of the sponge tissue The enzyme activities were measured and related to the concentration of deoxyribonucleic acid (DNA) Three general patterns of activity were noted among the enzymes found in measurable concentrations (1) activity proportional to DNA content (2) activity decreasing after the DNA had reached its maximum value and (3) activity continuing to increase after the DNA had reached a constant value (Table IV)

It is not possible at this time to correlate enzyme activities with properties of fibroblasts such as amino acid hydroxylation or cholesterol synthesis The presence of a large concentration of proteolytic enzymes

TABLE IV
PATTERN OF ENZYME ACTIVITY OF DEVELOPING CONNECTIVE TISSUE IN ADULT FEMALE RAT

Enzyme activity proportional to DNA content
Catalase
Prolinase
Phenolsulfatase
Cytochrome c reductase
Lactic dehydrogenase
Malic dehydrogenase
Glutamic oxalacetic transaminase
Enzyme activity decreasing after DNA reaches constant maximum
Peroxidase
Alkaline phosphatase
Enzyme activity increasing after DNA reaches constant maximum
β-Glucuronidase
Proteolytic (pH 3 2)
Prolidase
Acid phosphatase

after the level of DNA had become constant is of interest The enzymes in the first two categories of enzyme activity are probably related to the multiple metabolic activities of the fibroblasts while the proteolytic enzymes of the third category may be related to collagen degradation

III EFFECTS OF SEX AND TISSUE AGE UPON THE ACTIVITY OF THE FIBROBLASTS OF THE RAT

1 *Accumulation of Collagen in the Sponge Implant*

Sponge implants from 124 male and 139 female adult rats were used for this study The tissues were homogenized in saline and the insoluble residues were extracted with hot trichloroacetic acid (TCA) (5) The extracts were hydrolyzed in 6 *N* HCl and the collagen contents were calculated from the hydroxyproline values (14) Collagen accumulated rapidly during the initial 20 days of tissue age (Figs 3 and 4) Beyond 20 days the rate of collagen accumulation in the sponge of the female slowly decreased until the maximum collagen content was attained at approximately 40 days After 40 days there was a slow but constant decrease in collagen content which could be best expressed by a linear regression line with a significant negative slope ($p < 0\,01$) In the male sponge tissue (Fig 3) the rate of accumulation of collagen beyond day 20 decreased more rapidly than the rate in the female but the collagen content continued to increase to 300 days

The effects of the sex of the rat and tissue age upon the incorporation of lysine and its hydroxylation to hydroxylysine were studied by intraperitoneal injection of 12 microcuries (4 7 mg) of DL lysine 2 C^{14} per 100 gm body weight (19) Collagen was extracted from the fibrous protein fraction of the sponge tissue with hot TCA and the basic amino acids of the hydrolyzate of the extract were separated on Dowex 50 (12) and quantitated (13) Radioactivity in the eluate fractions containing lysine and hydroxylysine was determined Maximum hydroxylation occurred within 8 hours after the injection and remained constant for 24–26 hours (Fig 5) In subsequent experiments sponge tissues

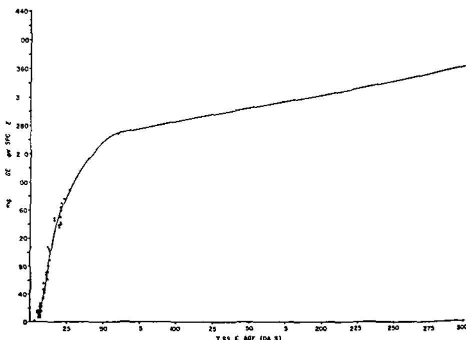


FIG 3 Relationship of collagen content to tissue age Sponge connective tissue from male rats was used

were removed 16 hours after the lysine injection i e during the period when a constant amount of labeled hydroxylysine was maintained Since hydroxylysine occurs only in collagen the introduction of C^{14} hydroxylysine would result in a specific labeling of the collagen molecule The equivalent amount of collagen was calculated on the basis of the experimentally determined percentage of hydroxylysine in collagen (1 24%) and the specific activity of the injected labeled lysine

FIG 4 Relationship of collagen content to tissue age Sponge connective tissue from female rats was used

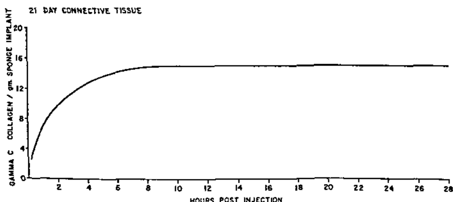


FIG 5 Appearance of C^{14} labeled collagen in the adult female rat

There was a marked sex difference in the amount of labeled hydroxylysine in the 12-day old tissue The tissue from the male had twice the amount found in the female tissue (Table V) Moreover a closely paralleled occurrence was noted in the *in vitro* studies The

TABLE V
INCORPORATION AND HYDROXYLATION OF C^{14}-LYSINE IN RAT CONNECTIVE TISSUE

Tissue age in days	Male		Female	
	C^{14} Hydroxylysine (μg /gm sponge)	Collagen equivalent (μg /gm sponge)	C^{14}-hydroxylysine (μg /gm sponge)	Collagen equivalent (μg /gm sponge)
13	0 39 (3)	31	0 19 (4)*	15
20	0 19 (8)	15	0 16 (8)	13
38	0 25 (1)	20	—	—
75	0 16 (2)	13	0 19 (2)	15
176	0 19 (2)	15	0 21 (1)	17
191	—	—	0 22 (1)	18
261	—	—	0 11 (2)	9

* Number of animals

TABLE VI
DISAPPEARANCE OF C^{14} LABELED COLLAGEN
YOUNG RAT CONNECTIVE TISSUE*

Sex of animal	Animal weight (gm)		Days post injection			
			0 67	7	14	23
			(μg /gm sponge)			
Male						
	1	315	17	17	13	—
	2	390	—	11	11	10
	3	355	40	17	28	27
	4	385	34	—	—	13
	Mean		31	15	17	17
Female						
	1	204	17	14	—	5
	2	227	12	10	—	4
	3	205	17	14	—	4
	4	200	15	9	—	7
	Mean		15	12	—	5
Female (Oophorectomized)						
	1	300	16	12	—	5
	2	341	14	16	—	5
	3	314	12	10	—	4
	Mean		14	13	—	5

Initial tissue age 12 days

supernatant fraction of the 12 day old sponge implant from the male rat hydroxylated twice as much labeled proline as did the supernatant from the female Since male and female tissues at day 12 were effecting a net synthesis of almost equal amounts of collagen (Figs 3 and 4) the male tissue must either have had a large amount of collagen de gradation occurring concomitantly with its greater synthesis or the ap pearance of labeled hydroxylysine in the male collagen reflected not only *de novo* synthesis but also nonsynthetic turnover of pre existing collagen

The rate of disappearance of labeled hydroxylysine from male and female 12 day old tissue was followed by removing sponges at 16 hours 7 14 and 23 days after lysine injection It can be seen (Table VI) that the male collagen rapidly lost labeled hydroxylysine in the first 7 days while the female collagen lost hydroxylysine at a slower rate This find ing supports the concept that the 12 day old male tissue had both a greater amount of synthesis and a greater degradation of collagen than the female tissue

Disappearance of labeled hydroxylysine from the female tissue oc curred at a constant rate so that only 30% remained after 23 days In the male following rapid loss during the first 7 days the disappearance of tagged hydroxylysine was negligible and after 23 days 50% remained There is a possibility that the male collagen may exist in two compart ments one undergoing rapid degradation the other being only slowly degraded

While collagen accumulation appeared to proceed at comparable rates in male and female tissue during the first 20 days of growth (Figs 3 and 4) the curves became widely divergent beyond 21 days the rate of accumulation in the male fell off more rapidly than the female To determine possible changes in the rate of synthesis beyond 20 days labeled lysine was injected into adult rats with sponge tissues of various ages and tagged hydroxylysine was determined 16 hours later A fairly constant amount of labeled hydroxylysine was found at all tissue ages within each sex (Table V) the female tissue obtained after 261 days of growth being the only exception

Data in Fig 3 indicated a greater rate of collagen accumulation in the male tissue at day 20 than at day 175 yet the amount of labeled hydroxylysine was not different at the two ages If the incorporation of isotope represented only *de novo* synthesis of collagen then a collagen degrading mechanism must have developed so that the interaction of synthesis and degradation resulted in a continually decreasing net syn thesis of collagen If the incorporation of isotope represented *de novo* synthesis plus turnover due to partial degradation or exchange there

must have been a gradual shift in the relative contributions of the two types of turnover so that by day 175 nonsynthetic turnover predominated

The female curve (Fig 4) from day 20 to its maximum at day 40 might be explained in the same manner as the male curve However there was a marked difference in the shape of these two curves the female curve rose at a more rapid rate and leveled off quite early Either the degradation mechanism came into play later in time but greater in magnitude than in the male tissue or else *de novo* synthesis gave way to other types of turnover much earlier than in the male

Beyond day 40 the collagen of the female tissue was gradually reduced *in contrast to the male collagen which continually increased* This suggests a fundamental sex difference in collagen turnover i e the female having a collagen degradation exceeding collagen synthesis At day 261 the incorporation of labeled amino acid into collagen in the female tissue had dropped from the level prevailing between days 13 and 191 suggesting a reduction in the activity of the fibroblasts in the older tissue Such a reduction would be in keeping with the change in the morphology of the fibroblast with tissue age i e the cell becoming narrower and exhibiting nuclear thinning and a decreased amount of cytoplasm

The available data do not permit the assignment of an unequivocal biochemical mechanism to explain the shapes of the collagen accumulation curves but there can be little doubt that there is a considerable metabolic turnover of collagen in the sponge connective tissues even of older ages It is interesting to note that the sponge tissue contains a number of active proteases and peptidases including prolidase and prolinase These enzymes reached their maximum levels after the rate of collagen accumulation had passed its peak and just at the time when degradation or turnover mechanisms must have become maximal

IV Effect of Gonadectomy Upon the Activity of Fibroblasts of the Rat

To elucidate the sex differences in the metabolism of connective tissue gonadectomy was performed in a series of animals Removal of the gonads caused a depression of some of the activities of the fibroblasts (Table VII) The amount of incorporation and hydroxylation of labeled lysine in collagen of 75 to 191 day old tissue was reduced to less than one half that of the tissue from intact animals Sterol synthesis from C^{14} acetate in slices of tissue from gonadectomized animals was also significantly reduced (16)

In oophorectomized rats the accumulation of collagen during the

TABLE VII
THE EFFECT OF GONADECTOMY UPON FIBROBLASTIC ACTIVITY IN RAT CONNECTIVE TISSUE

Sex of animal	µg C^{14} hydroxylysine per gm *sponge implant*	Labeled digitonin precipitable substance	
		cpm† *per gm* sponge implant	*standard deviation* of the mean
Female Intact	0 21 (4)‡	4 638 (35)	±3 228
Female Oophorectomized	0 09 (3)	2 596 (11)	±1 051
Male Intact	—	7 019 (30)	±3 785
Male Castrated	—	3 697 (6)	±2 381

16 hours post injection
† cpm counts per minute
‡ Number of animals

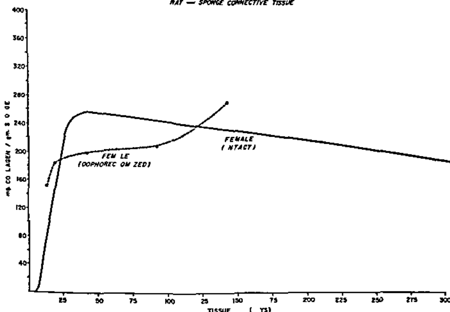


FIG 6 Relationship of collagen content to tissue age in oophorectomized and intact female rats

first 20 days following sponge implantation resembled that of the intact animals (Fig 6) The disappearance of labeled hydroxylysine in the 23-day period following C^{14} lysine injection at day 12 was unaffected by removal of the ovaries (Table VI) Beyond 20 days of tissue age

the accumulation of collagen in the sponge implants of 20 oophorectomized animals differed from that of the intact female and resembled that noted in the intact male i e a continued increase in collagen in the sponge with time The fact that collagen synthesis (hydroxylation of labeled lysine) was reduced in the older tissue from the spayed animals in spite of a net gain of collagen in the sponge suggested that the rate of collagen degradation had been reduced to an even greater extent than the rate of synthesis Apparently some substance from the ovary is intimately involved in the regulation of degradation of formed collagen in the sponge tissue

V The Relationship of Cholesterol to Connective Tissue

The fibroblasts synthesize a digitonin precipitable substance at an appreciable rate which is influenced by the sex of the animal The sterol is found in the ground substance in the fibroblasts as a part of the cell membrane and as a component of the fibrous protein Young collagen such as that found in the sponge biopsy contains a high mole ratio of sterol to collagen Collagen fibers prepared by repeated washings were found to have a 35 1 mole ratio of sterol to collagen Older collagen such as in the tendon of the rat tail had a 4 1 mole ratio of sterol to collagen It is known that skin contains a concentration of cholesterol which decreases with age (11) A collagen cholesterol complex in bone has been reported by Pikulev (20) the mole ratio calculated from his data indicates a much lower sterol value than for rat tail Examination of human aortic intima from 50 to 80 year old males and females indicated a 1 5 mole ratio of sterol to collagen (18) The relationship between sterol and fibrous protein is not clear sterol may be bound directly to the collagen it may be part of a complex involving collagen mucopolysaccharide and sterol or it may merely be a contaminant of the collagen preparation

The cholesterol of the ground substance from young connective *tissue is influenced by the level of serum cholesterol* (3) *Sterol* synthesized *in vitro* by the fibroblast is partially saline soluble and thus apparently complexed with protein of the ground substance The exact nature of the sterol complex in the ground substance is unknown Elevation of the serum cholesterol to abnormally high levels in rabbits caused a deposition of the sterol in young connective tissue of sponge implants (3) Cessation of the augmented cholesterol intake resulted in a fall in the serum cholesterol and a stabilization of the concentration of saline soluble cholesterol complex The cholesterol found in the saline insoluble material, however continued to increase presumably the result of tissue synthesis Entrance of cholesterol from the anoma

lously hypercholesterolemic serum to the sponge tissue was blocked by the administration of estrogen.

Older connective tissue such as that of the aortic intima of the adult rabbit, responded to the elevated serum cholesterol in a slower fashion. The amount of saline-soluble protein, presumably representing ground substance, was far less in the intima than in the sponge. The exposure of the intima for a protracted period of time to an elevated serum cholesterol resulted in the incorporation of cholesterol in both the ground substance and the saline-insoluble fraction.

VI. Properties of Fibroblasts Which May Be Related to Atherosclerosis

Vascular aging changes include a deposition of collagen in the intima and media, indicating a rate of synthesis exceeding that of collagen degradation. This is a dynamic process directly related to fibroblastic activity. Vascular atheromatosis is initiated by intimal irritation of a hemodynamic nature resulting in the expected response to injury, namely the appearance of fibroblasts.

Thus, vascular changes resulting from aging and atherosclerosis directly involve the connective tissues of the vessel. While any extrapolation from the connective tissue of the sponge to that of the vessel is open to serious criticism, nevertheless it must be recognized that these two connective tissues are largely composed of the same biochemical elements. While the metabolic turnover and reactivity of these elements may vary, the underlying mechanisms of collagen synthesis, the reactivity and synthesis of a portion of the ground substance, sterol synthesis, and enzymatic processes are all broad fundamental properties of the fibroblast. An understanding of the effects of sex of the donor tissue age, glandular ablation, and hormonal administration on these fundamental properties of the fibroblast should contribute significantly to the elucidation of some of the underlying mechanisms of aging and atherogenesis.

Acknowledgment

The authors gratefully acknowledge the assistance of Dr. Kung Ying Tang Kao during a phase of this research.

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Discussion

Dr Gross What do you call the collagen fraction?

Dr Boucek The sponge biopsy connective tissue was homogenized at high speed one minute in 0 15 M saline and filtered through cheesecloth The insoluble material was washed four times with saline and then hydrolyzed The hydroxy proline in the hydrolyzate was 11 to 14% of the total nitrogen

Dr Gross Do you feel you have a pure collagen?

Dr Boucek The purity of collagen can always be questioned

Dr Gross No I think there are reasonable criteria The key point is whether there is galactosamine as you imply within the collagen fibril and I wonder whether you do not have collagen plus something else that contains galactosamine What kind of an ultraviolet absorption curve do you get in an extract of this material?

Dr Boucek I do not have that information

Dr Gross How does the fat content change? Is the rise in fat different?

Dr Boucek Expressed on the basis of wet tissue weight total lipids increased significantly with tissue age

Dr Gross You don t think this difference in concentration of collagen is due to a greater increase in fat in the female than in the male? In other words is it a real difference?

DR. BOUCEK No We calculated the concentration of collagen on the basis of the weight of sponge implanted

DR WHITE Was the amount of collagen expressed on a fat free basis?

DR BOUCEK Collagen was extracted with hot trichloroacetic acid and the value expressed on the weight of the sponge that was implanted

DR WHITE Rather than on the weight of the total tissue?

DR. BOUCEK That is correct

DR GROSS Hot trichloroacetic acid extracts many things other than collagen Is this difference between male and female a real difference in the amount of collagen formed or just a reflection of a great increase in some other material in the female?

DR BOUCEK We appreciate that Dr Gross except that the percentages of hydroxyproline in the trichloroacetic acid extracts of the tissues from both sexes were similar

DR WHITE Do I understand you do hydroxyproline on an aliquot of the trichloroacetic acid extract? This extract is made to a volume so you have hydroxyproline on an aliquot? From that hydroxyproline concentration you calculate the collagen concentration? If you are extracting material which is nonhydroxyproline containing it does not affect the collagen determination

DR BOUCEK Yes that is right

DR GROSS If the hydroxyproline value is the same in both extracts that would be so

DR JACKSON Dr Boucek do you correct for the dilution of radioactive collagen by new collagen growth which occurred after you gave the C^{14} lysine? Because this need not be a disappearance If new material is laid down in the absence of C^{1} lysine this will lower the specific activity of the isolated hydroxylysine

DR BOUCEK The dilution of the value for labeled collagen in the sponge tissue would not be a factor since the values were expressed on the amount of labeled collagen per weight of the dry sponge which had been implanted Further the rapid accumulation of collagen in the sponge during the initial 20 to 40 days did not appear to influence the disappearance of tagged collagen since similar disappearance curves apparently exist in 100-day old tissues obtained from the female

DR WHITE Will you tell us about the methods applied to the hot trichloroacetic acid extract? This is hydrolyzed and the hydroxylysine and the lysine and histidine separated on the column? What do you do about possible labeled free amino acid which may be there? Do you not worry about that because you are measuring only the hydroxylysine activity? Do you measure only hydroxylysine fractions so these data are collagen calculated in terms of the hydroxylysine activity?

DR BOUCEK Yes Hydroxylysine was converted to its collagen equivalent

CHAIRMAN SMITH Of the list of proteolytic enzymes that you showed as being present in the sponge preparation none is known to attack collagen Four are peptidases which are rather specific for small substrates so I do not know that this bears any relationship to possible turnover Some years ago we tried to find enzymes in mammalian tissues at neutral pH which would attack collagen without any success whatever

DR GITLIN In what areas? Dont forget we have the problem here that was so clearly demonstrated by Dr Jackson that in some areas a demonstrable turnover of collagen does not occur in a given period of time What about bone?

DR BOUCEK I do not know about that

DR SHERRY Some years ago we showed that some spleen cathepsins will digest collagen at low pH s of 3 0 to 4 0 Of course at low pH the collagen becomes swollen probably making it more digestible This observation may be related to degradation of collagen *in vivo* If the local pH can be brought down far enough

DR GLIMCHER There is no reason to assume that it can t either

DR ADLERSBERG The rabbit is an excellent animal in which to produce atherosclerosis the dog is not Serum lipid patterns differ considerably between the species I would like to know whether there are any established chemical or physicochemical differences between the various species in the behavior of collagen I noticed in one of Dr Gross slides there was a difference between the collagen obtained from calf skin and guinea pig skin in relation to the gelation of collagen solutions

DR GROSS There are differences chemically and physicochemically even in the same animal I have looked at the collagen of the carp scales skin and swim bladder If gelatin is made from all three the composition in terms of glycine proline hydroxyproline is about the same but in the whole collagen as pure as it can be made a difference is found in the amount of these amino acids but not in their proportions relative to each other

What does this mean? These different collagens are mixed with something else The s ale collagen is mixed with something which makes it resistant to collagenase The carp swim bladder collagen will go into solution in dilute acetic acid whereas the skin collagen will not Amounts of tyrosine associated with collagens from *different tissues of the same animal vary widely as do the amounts* of sugars

It is often hard to tell whether the differences in properties and composition are due to a substance in fortuitous association with the collagen or to intrinsic differences in the collagen molecule

DR. BERLINER Dr Dougherty and I have demonstrated that tissue culture fibroblasts can synthesize cholesterol from acetate also that fibroblasts are very active in metabolizing the steroid hormones I believe the most active cells in the body with regard to steroid metabolism are the fibroblasts especially hydrocortisone and progesterone Dr Boucek mentioned that estrogen would deplete the cholesterol in blood I wonder if he knows where this cholesterol is going

DR BOUCEK Estrogen administration while the rabbit was receiving an augmented (2%) cholesterol intake did not influence the level of cholesterol in the serum but blocked its entrance into the young sponge connective tissue

DR ADLERSBERG Do the corticosteroids block the entrance of the cholesterol into the tissue?

DR BOUCEK I do not know

DR JACKSON Regarding species difference my surgical colleagues tell me nothing at all happens when sponges are implanted into the human

DR BOUCEK That is incorrect We have done a large study of human connective tissue obtained by the sponge implantation technique The guinea pig appears to be slow in the development of connective tissue in the sponge

DR JACKSON Again my colleagues tell me that when dogs are fed high cholesterol diets atherosclerosis does not result But if granular tissue is stimulated in the aorta a high content of cholesterol appears I wonder whether some of this was actual absorption from plasma as well as synthesis

DR BOUCEK Unquestionably young connective tissue—and I think the pathologists have pointed this out—will absorb cholesterol and from the information we have the fibroblasts will synthesize cholesterol

DR. JACKSON Is there any stainable lipid in your sponge tissue?

DR BOUCEK Yes

DR JACKSON In the granuloma we find the same situation Carrageenin granuloma tissue has a tremendous amount of lipid present Complete analysis of all the fatty acids gives us a result identical with that of the plasma lipids Dr James at the National Institute of Medical Research did this for me With usual histological methods you cannot see lipid present in the granuloma so we assume that this must be attached to something else which protects it from the Sudan dyes

DR ADLERSBERG Did you determine any other lipids besides cholesterol that is triglycerides or phospholipids? If so what did you find?

DR BOUCEK Augmented cholesterol feeding caused a sharp rise in total lipids and phospholipids as well as cholesterol in the tissue Removal of the added cholesterol from the diet resulted in a plateauing of the total lipids and a decrease in phospholipids and neutral fat of the connective tissue in the sponge However the cholesterol continued to increase

DR HARTROFT In your feeding experiment what was the basal diet? Chow?

DR. BOUCEK The regular laboratory chow for rabbits (Purina chow) supplemented with cholesterol

DR ADLERSBERG Was the cholesterol fed as such or in a fat medium? In the latter case in what was it dissolved?

DR BOUCEK It was dissolved in ether and then mixed with the chow

Mechanisms of Blood Coagulation*

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Whatever the role that blood clotting may play in the pathogenesis of atherosclerosis no one doubts the importance of this process in the production of those changes which result ultimately in the patients difficulties In patients with coronary arterial disease for example thrombosis is the most frequent cause of sudden complete obstruction of a coronary vessel and endocardial thrombi a source of crippling emboli occur in about half the cases of myocardial infarction (16) It seems appropriate therefore to review some recent experimental insights into the pathogenesis of thrombosis The available literature is of course boundless and it would take far longer than the time allotted for this entire conference to do justice to the current status of this problem I think it would be more profitable to deal only with a restricted area I shall concern myself principally with the results of some studies performed by my associates and myself first at the Johns Hopkins University and during the last eight years at Western Reserve University School of Medicine

We hear a great deal about how blood clots but little attention is paid to the more remarkable problem—how it is that blood which clots so readily when it leaves the vascular tree normally remains fluid within our vessels? What are the mechanisms which keep this unstable system liquid so that we can maintain the circulation? I do not have a clear answer to this question but the beginnings of an answer are appearing as a strange disorder named Hageman trait is studied

About five years ago a patient was referred to University Hospitals of Cleveland because his physicians had observed that the clotting time of his venous blood was greatly prolonged The patient Mr John Hageman had a peptic ulcer for which an operation was contemplated the clotting time had been performed as a routine measure A careful review of Mr Hagemans previous history revealed no evidence of a bleeding tendency despite the usual injuries operations and dental ex

The original investigation described in this review was supported in part by a research grant (PHS H1661C5) from the National Heart Institute of the National Institutes of Health and in part by a grant from the Cleveland Area Heart Society

tractions There was no family history of bleeding However, the clotting time of his venous blood was indeed greatly prolonged—prolonged to the degree which one associates with the severest cases of hemophilia (22)

Miss Joan Colopy and I then performed studies to localize the abnormality responsible for the prolonged clotting time When the plasma was separated from the blood cells by centrifugation the prolonged clotting time was found to be associated with a defect in Mr Hageman s plasma Normally plasma which has been depleted of its cellular elements by centrifugation at high speeds clots readily when it is placed in glass tubes Mr Hageman s plasma on the other hand failed to clot under these conditions Presumably then Mr Hageman s plasma shared the defect observed in his whole blood

The abnormality in Mr Hageman s plasma was then localized to the earliest step in the clotting process the development of thromboplastic activity The process of coagulation can be divided into three general steps The initial stage of the clotting process consists in the development of thromboplastic activity This thromboplastic activity then converts prothrombin into thrombin The thrombin in its turn converts fibrinogen into the solid fibrin clot In Mr Hageman the first stage the development of thromboplastic activity was defective

A number of different substances have been implicated in the development of thromboplastic activity in cell poor plasma These include antihemophilic factor the substance lacking in classic hemophilia (15) Christmas factor or plasma thromboplastin component (1, 4 29), the substance lacking in Christmas disease and plasma thromboplastin antecedent or PTA (28) a substance thought to be lacking in certain patients with mild hemorrhagic symptoms In turn it was possible to show that Mr Hageman s plasma lacked none of these components Mr Hageman s disorder appeared to be unique

Next a fraction of normal plasma was prepared which corrected the defect in Mr Hageman s plasma This fraction contained none of the previously described clotting factors A similar fraction was prepared *from Mr Hageman s plasma This "new substance was called* Hageman factor to avoid using a term which implied that we understood how it worked A deficiency of this factor was called Hageman trait thus the blood of patients with Hageman trait is deficient in Hageman factor

Hageman factor had one interesting property When small amounts of partially purified Hageman factor were added to normal blood or plasma, it greatly accelerated clotting. In other words Hageman factor behaved as if it were normally in an inactive state When it was purified it was in some way activated so that it became a potent accelerator of

clotting Indeed its potency was such that it appeared to account for some of the puzzling properties of other supposedly purified clotting factors For example some years ago it was suggested that clotting was initiated by the action of plasmin or fibrinolysin This hypothesis was discarded when it became evident that the preparations of plasmin which promoted clotting were contaminated with something which has since been identified with Hageman factor (23)

How does all this explain what maintains the fluidity of blood in the body? For an understanding of this we must turn to the early part of this century when Jules Bordet studied the effect of glass surfaces on blood (5) He found that plasma, prepared from mammalian blood by high speed centrifugation would not clot when kept in paraffin lined vessels but clotted quite promptly in glass tubes This plasma was deficient in the cellular elements of the blood Presumably then glass initiated clotting by reacting with some component of the plasma The clot promoting effect of glass upon cell poor plasma is easily demonstrable Cell poor plasma was prepared so that it did not come in contact with glass After recalcification the plasma remained fluid for more than 2 hours However when this same plasma was placed in glass tubes it clotted in 14 minutes In other words contact with glass greatly accelerated the clotting of normal cell poor plasma

One by one the various components of the plasma which are involved in clotting have been implicated in the action of glass and one by one they have been discarded for lack of convincing evidence A solution now seems at hand Shafrir and de Vries (30) in Israel and Margolis (12 13) in England recently demonstrated that the action of glass requires the presence of some substance in plasma which they could not identify with previously described clotting factors Although at the time they did not test for this possibility it was evident from their studies that the effect of glass might require the presence of Hageman factor Stimulated by these reports Dr Jerold Rosenblum and I (25 26) tested the effect of glass upon a wide variety of plasmas We have confirmed the presence of a specific substance in human plasma which promotes clotting when plasma comes in contact with glass Plasma devoid of cells was prepared in silicone coated vessels from blood obtained from a normal individual and from a patient with classic hemophilia Normal plasma alone kept in silicone coated tubes did not clot within the period of observation Similarly hemophilic plasma was incoagulable whether or not it was treated with glass However a mixture of glass treated hemophilic plasma and normal "silicone plasma clotted within 18 minutes In other words treatment of hemophilic plasma with glass produced changes such that it now

accelerated the clotting of normal plasma Hemophilic plasma behaves as if it is deficient in a specific clotting factor antihemophilic factor One may conclude from this experiment therefore that the clot promoting effect of glass did not require the presence of antihemophilic factor

In the same way it has been shown that treatment with glass produced clot promoting activity in plasma deficient in all the known clotting factors—that is all but one When plasma obtained from patients with Hageman trait was tested no clot promoting activity arose Plasma deficient in Hageman factor was tested by the same method used with other pathologic plasmas The plasma was shaken with glass and the glass was then removed by centrifugation This glass treated plasma was then mixed with normal plasma in silicone coated tubes The mixture failed to clot within 2 hours Unlike all other plasmas tested glass treated plasma deficient in Hageman factor lacked clot promoting properties These experiments suggest that the clot promoting effect of glass requires the presence of Hageman factor

What does glass do? Following a suggestion of Fiala (7) we observed that a number of substances which—like glass—are good adsorbents accelerated clotting in the same way For example barium carbonate kaolin charcoal and Super cel are all clot promoting agents and all require for their activity the presence of Hageman factor These observations make it seem likely that glass acts by removing an inhibitor of Hageman factor We have not been able to isolate such an inhibitor from normal plasma However plasma obtained from patients with Hageman trait will inhibit the action of glass upon normal plasma In one such experiment plasma deficient in Hageman factor was treated with crushed glass As in the previous experiments this glass treated plasma did not clot in silicone coated tubes in 2 hours Normal plasma treated with glass clotted in 12 minutes Next normal plasma and plasma deficient in Hageman factor were mixed together The mixture of the two plasmas was then treated with glass The mixture clotted in 29 minutes in silicone coated tubes In other words plasma deficient in Hageman factor inhibited the clot promoting effect of glass However once glass had been allowed to exert its effect on normal plasma plasma deficient in Hageman factor did not appreciably inhibit the effect of glass Normal plasma and plasma deficient in Hageman factor were treated separately with glass The two glass treated plasmas were then mixed This mixture clotted in 14 minutes In other words once glass had been allowed to react with normal plasma the addition of plasma deficient in Hageman factor did not inhibit clotting Moreover the inhibitory property of plasma deficient in Hageman factor could be sharply reduced by treating such plasma with glass These experiments

demonstrate that plasma deficient in Hageman factor inhibits the clot promoting effect of glass These experiments suggest that the initiation of clotting then may consist in the removal of an inhibitor from plasma freeing Hageman factor This factor in its turn starts the clotting process

Teleologically it is hard to believe that so potent a clot accelerator as Hageman factor could be allowed to remain free in the blood for long If some active Hageman factor were accidentally liberated intra vascularly extensive thromboses might result This teleological argument may have some merit Margolis (12 13) demonstrated that the clot promoting agent which appears when plasma is treated with glass is un stable and its activity diminishes when the plasma is separated from glass Margolis attributed this diminution of clot promoting activity to an antagonist in plasma The decline in clot promoting activity is readily demonstrable The clot promoting activity of glass treated plasma was measured by its effect on the clotting time of plasma which had been kept in silicone Again the silicone" plasma alone remained fluid for more than 2 hours When plasma which had been freshly treated with glass was added to it the mixture clotted in 16 minutes If how ever the glass treated plasma was first incubated at 37°C for 2 hours it shortened the clotting time of the silicone plasma only to 41 min utes Thus the clot promoting activity of the glass treated plasma had deteriorated This experiment supports the view that the clot promoting activity induced by glass is labile

As we have said there is strong evidence that the clot promoting substance which evolves when plasma is treated with glass is Hageman factor Since the clot promoting agent is inactivated by plasma Hage man factor too should be inactivated by plasma Experiments were per formed which demonstrated that plasma does indeed inactivate Hage man factor (21 26) A concentrate of Hageman factor was mixed with silicone plasma The silicone plasma alone did not clot within an hour At first an aliquot of the mixture of Hageman factor and silicone plasma clotted in 7 minutes That is Hageman factor greatly accel erated the clotting process Another aliquot of Hageman factor and silicone plasma was incubated for 2 hours and then tested Now the mixture clotted in 26 minutes In other words the clot promoting ac tivity of the Hageman factor had deteriorated during its incubation with normal plasma This experiment confirms the hypothesis that Hage man factor and the clot promoting agent in glass treated plasma are identical

Next it was possible to separate the fraction of plasma which in activates Hageman factor from other components of the clotting mech

anism We are now in the process of trying to purify this inactivating substance it is probably either an α or β_1 globulin but I am still most uncertain about this In any case the agent which inactivates Hageman factor acts as if it is an enzyme (21) A crude fraction containing the inactivating factor was prepared by precipitation with ammonium sulfate The effect of this crude preparation upon purified Hageman factor was then tested A mixture of the crude inactivating factor and Hageman factor was incubated at various temperatures The clot promoting activity was measured immediately after the factors were mixed and at successive intervals The mixture of Hageman factor and the inactivating agent gradually lost its clot promoting activity The rate of loss was a function of temperature When Hageman factor and its inactivator were incubated at 37°C the loss of clot promoting activity was more rapid than at 25°C At 1°C no measurable deterioration of Hageman factor occurred during the period of observation The rate of deterioration at 25° and 37°C seemed to follow the kinetics of enzymatic reactions It was not possible to identify the inactivating property with plasmin the proteolytic enzyme of plasma active at neutrality nor have other substrates of this enzyme been found

These studies of patients with Hageman trait thus led to the following hypothesis the fluidity of blood is maintained at least in part by inhibitors directed against Hageman factor Exposure of blood to glass converts Hageman factor to an active form by releasing it from inhibition The active Hageman factor then initiates clotting Once active Hageman factor is destroyed enzymatically a device which may prevent its continued action Plasma then may contain two types of inhibitory mechanisms related to the initiation of clotting one which prevents the activation of Hageman factor and the other which destroys Hageman factor

I have carefully avoided any implication that these mechanisms may be involved in the formation of intravascular thrombi Concerning this possibility only speculation is possible at this time Perhaps if the endothelial lining of blood vessels were to lose its silicone like properties and acquire glass like properties the injured area could serve as a focus at which Hageman factor might become active and initiate the clotting process Figure 1 is a drawing taken from a paper by Moolten and his associates (14) They injected air intravascularly into rabbits and then examined the mesenteric vessels immediately after death At first no meniscus could be seen within the vessels This is characteristic of fluid in contact with a surface such as silicone Then as endothelial death occurred a meniscus gradually appeared as if the surface of the vessels was now wettable or glass like At this time no histologic altera

tions were noted by the usual techniques Perhaps then minor alterations in the endothelial lining may provide the necessary stimulus for promoting intravascular clotting. It is not hard to imagine that similar changes might occur upon injury to the endothelium covering an atheromatous plaque and be responsible for the initiation of thrombosis

I have concentrated my remarks on the effect of glass upon Hageman factor None of these studies negate the possibility that glass also alters other clotting factors Indeed this is almost certainly the case However

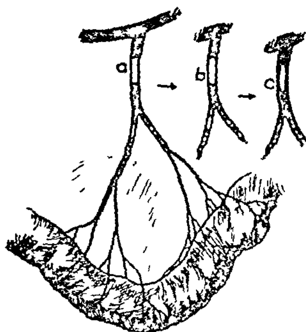


Fig 1 Development of endothelial wettability following cessation of circulation as indicated by the formation of a concave meniscus between the blood and the air a, Appearance of an air segment in a mesenteric vein shortly after the death of an experimental animal b eighteen minutes after death c forty five minutes after death Sketch is from actual experiment showing how the initial slight concavity of the meniscus between blood and air is masked when in the direct line of vision but becomes clearly defined as the film of blood advances along the uncovered endothelium with increasing wettability From Moolten *et al* (15)

the data previously recorded in the literature must now be reappraised in the light of the studies I have described today

I have deliberately spent all this time on what may be the initial step in the clotting process I should like now to take a hop skip and jump through the rest of the clotting mechanism (Fig 2) Besides

Hageman factor antihemophilic factor Christmas factor platelets and calcium ions are needed for the optimal development of thromboplastic activity *in vitro* The order in which these substances act and the role each plays is unclear although many suggestions have been made in the last few years We do not know upon which of the components of the clotting system Hageman factor works this problem is under active study in our laboratory In the test tube crude extracts of the phosphatides of brain or soy beans may be substituted for platelets in the development of thromboplastic activity Just last week Troup and Reed (33) reported that the clot accelerating property of platelets is related to its content of phosphatidyl serine

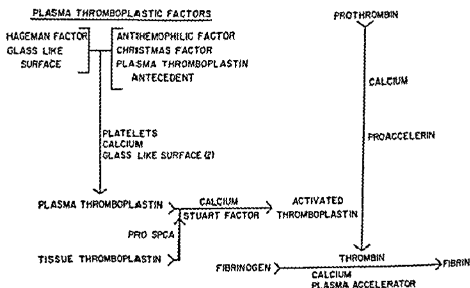


FIG 2 A simplified outline of the process of blood clotting Inhibitors have been described which act at various stages in the clotting process Strong evidence exists that proaccelerin is converted to an active form accelerin prior to its utilization

Of course the classic source of thromboplastin is not the plasma but animal tissue Extracts of brain or lung have long been used to initiate clotting in the test tube and may play an important role in the control of bleeding at site of injury Although platelets have been implicated as a source of thromboplastin it has been demonstrated repeatedly in recent years that they are in fact a poor source of this substance (17 34) Tissue thromboplastin by itself is actually ineffective it must first react with what Dr Alexander has called the precursor of serum prothrombin conversion accelerator (or SPCA) (2) The product of this

reaction then appears to behave in the same manner as the thromboplastin which has evolved from the plasma

Next thromboplastin whether it has arisen from plasma or tissue must react in some manner still not clear with two other substances before it can convert prothrombin to thrombin at a maximal rate Probably the first of these in terms of its place in the clotting scheme is Stuart factor a stable component of plasma recently delineated independently by several groups (8 32) Stuart factor and pro SPCA were formerly considered to be a single entity Factor VII or proconvertin The second substance with which thromboplastin may react is proaccelerin or labile factor (to mention but two of its many names) good evidence exists that this substance must first be converted to an active form accelerin before it can act it is probable that this conversion is effected by thrombin The product of these successive reactions then converts prothrombin into thrombin and this in turn so alters fibrinogen that it forms an insoluble substance fibrin Sherry and Troll (31) demonstrated that thrombin like such known proteolytic enzymes as trypsin and plasmin is a hydrolytic enzyme digesting the synthetic substrate paratoluenesulfonyl L arginine methyl ester A little earlier than this Lorand and Middlebrook (11) and Bettelheim and Bailey (3) demonstrated that thrombin splits fibrinogen into small polypeptide fragments and a larger molecule which may be designated as soluble fibrin In turn the soluble fibrin polymerizes to form the insoluble fibrin clot The conversion of fibrinogen to fibrin by thrombin will take place in the absence of bivalent cations However calcium ions in the concentration present in human blood accelerate the formation of fibrin about three fold (24) make the resultant fibers more resistant to the action of acids bases and urea (10 27) and increase their tensile strength (6 27) The conversion of fibrinogen to fibrin is also accelerated by some unidentified factor in plasma (19) this acceleration may be due to a nonspecific colloidal effect of the plasma proteins (9)

Next I should like to encroach for one moment upon Dr Sherry and Dr von Kaulla s topic There is evidence that the clotting process accelerates the activation of plasmin by streptokinase or chloroform (18) and indeed that clotting may accelerate the spontaneous activation of plasmin (18 20) Thus a mechanism may be provided for the removal of small intravascular clots distinct from the classic processes of organization and recanalization The mechanism of this activation has been studied by Dr Sherry who will I hope discuss his observations

Finally you will note that I have omitted from this breezy discus

Hageman factor antihemophilic factor Christmas factor platelets and calcium ions are needed for the optimal development of thromboplastic activity *in vitro* The order in which these substances act and the role each plays is unclear although many suggestions have been made in the last few years We do not know upon which of the components of the clotting system Hageman factor works this problem is under active study in our laboratory In the test tube crude extracts of the phosphatides of brain or soy beans may be substituted for platelets in the development of thromboplastic activity Just last week Troup and Reed (33) reported that the clot accelerating property of platelets is related to its content of phosphatidyl serine

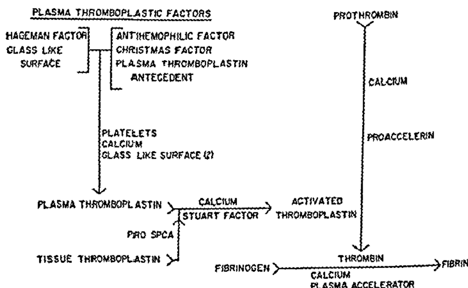


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Role of Blood Coagulation and Fibrinolysis in the Pathogenesis of Arteriosclerosis*

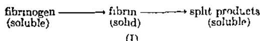
Tage Astrup

Biological Institute Carlsberg Foundation Copenhagen Denmark

The results of recent studies on the dynamic balance between fibrin formation and fibrinolysis have made it possible to begin speculation about the physiological significance of these processes This has led to the formulation of a concept of the pathogenesis of arteriosclerosis (9) which apparently confirms and extends the hypothesis of Duguid (21 23) Since Duguid's hypothesis constitutes a break with most of the prevailing ideas of the pathogenesis of arteriosclerosis (and atherosclerosis) (27) it is perhaps significant that we have arrived at a similar concept from a completely different background My chief purpose in this presentation is to describe how this concept was developed

I The Hemostatic Balance

There exists in the organism a dynamic balance between fibrin formation and fibrinolysis (I)



(I)

Fibrinogen has a high turnover rate in the circulating blood which indicates that it is rapidly metabolized Various authors give a half life of about 6 days compared to 13–21 days for albumin and 15–20 days for γ globulin Prothrombin and platelets also disappear rapidly from the blood and the concept of blood coagulation as a dynamic process which is continuously in action has been stressed especially by Allen (5 6) It is interesting and perhaps of significance for the problem of the pathogenesis of atherosclerosis that the β lipoproteins also have a high turnover rate in blood of about 4 days

The disappearance of fibrinogen from the blood is presumably caused by its transformation into fibrin by the highly active blood clotting system The latent activity of this system permits a continuous formation of clotting agents which transforms fibrinogen into fibrin The fibrin dis

Aided by grants from the Josiah Macy Jr Foundation New York and the National Danish Association against Rheumatic Diseases

sion such problems as the mechanisms and role of clot retraction the formation of fibrinoid and the fashionable topic of the effect of lipids exercise emotions and so forth on the rate of coagulation Indeed, there is no end to the possible subjects which one might include but if there is no end to these there should at least be an end to this talk

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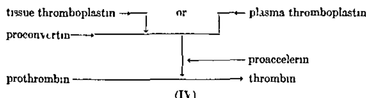
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See Discussion (page 279)

whom blood coagulation is deficient Hemostatic balance has been discussed elsewhere (11) but a few comments will aid in the understanding of its role

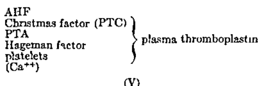
Injury of a tissue releases tissue thromboplastin which produces thrombin from prothrombin aided by two other plasma factors proaccelerin (Ac-globulin factor V) and proconvertin (factor VII) Deficiencies in proaccelerin or proconvertin can cause a hemorrhagic diathesis indicating the significance of the tissue thromboplastin for normal hemostasis and normal wound healing (scheme IV) In this scheme the effects of the Stuart Prower factor are not included

Prothrombin can also be converted into thrombin by a thromboplastic agent which can be formed in plasma but this plasma thromboplastin apparently acts without proconvertin (scheme IV)



(IV)

Plasma thromboplastin is formed from precursors in the blood by complicated reactions involving many components of which those shown in scheme V are known

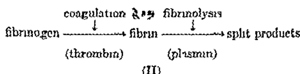


(V)

Lack of any of these components can produce a bleeding disease This applies also to a deficiency in the Hageman factor although this factor differs slightly from the other components since most of the persons lacking it do not present clinical symptoms of a hemorrhagic diathesis However we have recently in Copenhagen observed patients with a bleeding disorder which apparently is caused only by lack of Hageman factor (35) so that we think that all factors of the plasma thromboplastin system have in principle the same effect Bleeding disorders in this group comprise cases of thrombocytopenia and the whole group of hemophilioid diseases

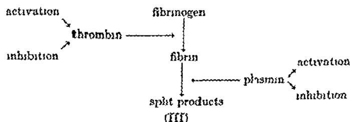
These diseases demonstrate the significance of the plasma thromboplastin system for normal hemostasis and normal wound healing Blood from this group of patients will produce normal clots when tissue thromboplastin is added

solving system in blood has also a high latent activity which makes possible a continuous formation of small amounts of fibrinolytic enzyme. The amount of fibrin present therefore depends upon the activity of the coagulation system as well as the activity of the fibrinolytic system. If fibrin is not removed with the same velocity as it is formed an accumulation would occur. Since this is known only from pathological cases, we assume that the balance between fibrin formation and fibrin resolution normally regulates the amount of fibrin deposited. For the regulation of this balance we have the scheme shown (II).



(II)

The active agents in this balance are the clotting agent thrombin and the protease plasmin. The velocity of fibrin formation therefore depends upon the concentration of thrombin, and the velocity of fibrin dissolution depends upon the concentration of plasmin. However, thrombin and plasmin are not present free in the blood. Both agents have to be formed by means of activating agents from precursors circulating in the blood. Furthermore, both these agents as well as their activators are inhibited by a number of inhibitory compounds. This means that the available concentrations of thrombin and plasmin depend upon the activity of activating and inhibitory systems, so that we end up with the scheme shown (III) for the hemostatic balance in the organism.



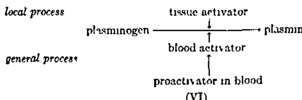
(III)

This balance is apparently a dynamic balance between the effects of a number of simultaneous reactions. We will consider the significance of some of these reactions in the total balance, although the inhibitory systems will not be included because of the limited knowledge of these processes.

II. The Coagulation System

The significance of the clotting process for normal hemostasis is apparent from the occurrence of hemorrhagic disorders in patients in

Plasmin is also developed in blood by complicated systems (8 9) which involve activation by means of an activator in tissues or by formation of an activator in blood (scheme VI)



The formation of high concentrations of the activator in blood occurs in cases of shock and in a few other pathological conditions This activator can be carried with the blood stream to all places in the organism We therefore assume that this process is of general significance to the organism For local tissue repair we assume that the tissue activator is of greatest importance since it is a fairly insoluble substance which is contained in the particulate matters in the cells (17)

Recently we have developed a method for the quantitative extraction and estimation of the tissue activator (13) We have studied the concentration of the tissue activator in a number of animal and human organs Table I shows the concentration of tissue activator in some human organs (4) Average values are estimated in units of an activator preparation made from pig heart and used as a standard The estimations show large variations from organ to organ Unfortunately the individual variation of samples of the same organ from different persons is also great However the organs can be divided into groups of high medium or low activity It is interesting that high concentrations of plasminogen activator are found in those organs which show a tendency to bleed especially in the uterus the adrenals the lymph nodes the

TABLE I
Tissue Activator Concentration in Human Organs

Organ	Average concentration (units per gram fresh tissue)	Organ	Average concentration (units per gram fresh tissue)
Uterus	720	Kidney	119
Adrenals	410	Muscle	110
Lymph node	378	Heart	82
Prostate	334	Brain	35
Thyroid	325	Testes	25
Lung	223	Spleen	20
Ovary	210	Liver	0
Pituitary	140		

When a tissue is injured hemostasis is produced by the release of tissue thromboplastin and aided by activation of the plasma thromboplastin system The powerful effect of this process in producing local hemostasis is demonstrated by the fact that surgery can be performed safely on patients under dicumarol therapy (20% prothrombin activity) provided the prothrombin level is controlled by an accurate estimation method (16 32 38) This principle was introduced in lung surgery and in heart surgery for mitral stenosis Even vascular surgery (grafting) could be performed under dicumarol prophylaxis (37) These results indicate that normally the clot promoting system which produces local hemostasis is considerably overbalanced and they give theoretical support for the use of drugs of the dicumarol type in the long term treatment of thrombosis

However at sites where the amount of tissue thromboplastin released is low in comparison with the amount of extravasated blood or in tissues low in tissue thromboplastin hemostasis depends solely upon the effect of the plasma thromboplastin system This is true in larger wounds where tissue thromboplastin has been washed away We have recently found that the joint tissues (synovial membrane and fibrous capsular tissue) contain very little tissue thromboplastin so that local hemostasis in joints depends completely upon the plasma thromboplastin system (18) Hemophilic patients usually get their hemorrhages in the joints since it is the plasma thromboplastin system which is deficient in these patients On the other hand the long periods of remission in hemophilic patients are probably chiefly caused by the effect of the tissue thromboplastin in hemostasis of minute injuries in other tissues

III The Fibrinolytic System

The role of fibrin in normal hemostasis is apparent from the hemorrhagic disorders recorded in patients with afibrinogenemia Copley (19) suggested that fibrin forms a normal constituent of the endothelial lining and that it helps to retain the integrity of the vascular system especially the capillaries In fact so called fibrinolytic purpura would be difficult to understand if fibrin did not form a normal component of the vascular system The occurrence of fibrin like fibrils on the endothelial surface has recently been demonstrated by electron microscopy by Roos (33)

Excess of fibrinolytic activity can produce a hemorrhagic diathesis i e in obstetrical shock in prostatic cancer and in a few other conditions These observations demonstrate the significance of the other side of the hemostatic balance viz the resolution of fibrin effected by plasmin

We will now consider what occurs in the organism if the balance between fibrin deposition and fibrinolysis is shifted so that too much fibrin is formed or too little is redissolved We have recently pointed out that there are many reasons for considering the fibrinolytic system as one of the most significant mechanisms by which the organism regulates tissue repair and connective tissue formation in wounds (7 9)

The constant infliction of minute injuries in daily life necessitates the formation of minute deposits of fibrin in the healthy organism as part of normal tissue repair The re establishment of normal conditions therefore depends upon the ultimate removal of these fibrin deposits after wound healing If excess fibrin is left in a wound for a longer period excess formation of connective tissue will occur This is seen in the normal migration of fibroblasts into a fibrin clot followed by organization of the clot finally transforming the thrombus into connective tissue This process is part of a normal wound healing

Naturally this process can be hampered by decreasing the amount of fibrin produced in tissue injury and this is in fact the basis for the treatment with dicumarol which decreases the effectiveness of the blood clotting system and therefore delays the deposition of new amounts of fibrin We have seen that local hemostasis is still sufficient for normal wound healing in these cases but the therapy is effective only so far as it prevents formation of fibrin For the resolution of already formed fibrin clots the organism depends upon its fibrinolytic system A characteristic example of this is seen in the crippling joints after hemorrhage in hemophilic persons (10) Since hemostasis in the joint tissues depends upon the activity of the plasma thromboplastin system because of their low concentration of tissue thromboplastin (18) larger amounts of blood will escape from the vessels before clotting takes place The large fibrin deposit which forms if the blood is not removed can only be redissolved with difficulty by the available fibrinolytic activity Hence there will result a formation of a larger amount of connective tissue than during healing of a joint lesion in a normal person and the end result is crippling of joints characteristic of hemophilic patients

An indication of the significance of the balance between fibrin deposition and fibrinolysis for the regulation of connective tissue formation is also probably found in the prevalence of fibrosis in some of the organs in Table I with little or no fibrinolytic activity (e g liver spleen and others)

When we now turn to the processes in the vessel walls we must also consider what happens if fibrin is deposited on their surfaces It would be difficult to imagine that injury should not occur as a normal process in the walls of vessels caused by infection by strain or by other means

prostatic gland and the thyroid We assume this indicates the role of the tissue activator in regulating local hemostasis and wound healing

At the other end of Table I we find little or no activity in a number of organs Liver is totally free from tissue activator It is tempting to see a connection between the function of the liver in the production of blood proteins probably including plasminogen and the absence of plasminogen activator in the tissues Perhaps Nature has had to make a compromise on this point since it can easily be envisaged that the presence of plasminogen activator in a plasminogen producing organ could be dangerous to the organism

Bleeding in the adrenals can be explained as thrombosis followed by release of large amounts of plasminogen activator from the necrotized tissue dissolving the thrombus and so leading to hemorrhage

So far the frequent occurrence of cerebral hemorrhage has not been explained Local hemostasis should be more than sufficient in this organ in view of its extremely high thromboplastic activity and also the results previously mentioned of surgical intervention in patients under dicumarol therapy During the past few years cerebral thrombosis has been treated by administration of dicumarol similar to the long term treatment of patients with coronary thrombosis The rationale of this therapy is apparently based on the low fibrinolytic activity in the brain tissue according to Table I which should not interfere with the effectiveness of local hemostasis and wound healing However we have recently found that the brain membranes contain an extremely high concentration of plasminogen activator (31) This is especially true of pia mater which in animals has activities up to 4000 units per gram and thus constitutes the tissue with the highest concentration of plasminogen activator yet encountered in our investigations Since many of the cerebral arteries pass through the pia mater thrombosis in any of these arteries can produce necrosis and a release of plasminogen activator thus transforming the thrombus into a site of hemorrhage We think therefore that the use of dicumarol therapy in the treatment of cerebral thrombosis should be reconsidered

IV The Pathogenesis of Arteriosclerosis

It is apparent from the above data that the balance between fibrin deposition and fibrinolysis regulates normal hemostasis Deficiencies in the blood clotting system as well as excess activity of the fibrinolytic system can produce hemorrhage and thus prevent normal wound healing However local hemostasis is normally very effective and even over balanced in most organs except in those with a low thromboplastic activity (joints) or a high fibrinolytic activity (see Table I)

patients in apparent contradiction to the role attributed by Duguid to mural thrombi in the pathogenesis of arteriosclerosis (36) Since the clotting time in hemophilia is greatly prolonged and fibrin deposition therefore is impaired it seemed paradoxical that fibrin deposition followed by development of arteriosclerosis could occur in hemophilic patients We have recently had an opportunity to discuss this puzzling phenomenon (10) Since the intima according to our results has an extremely high thromboplastic activity and since tissue thromboplastin acts on hemophilic blood just as well as on normal blood there is no reason to assume that the deposition of fibrin on an injured intimal surface will not occur almost as frequently and as rapidly in hemophilic patients as in normal persons Hemophilic patients are deficient in the plasma thromboplastin system and it is the normal effect of tissue thromboplastin on hemophilic blood which has delayed the elucidation of this hemorrhagic disorder for so long We have also been able to explain joint bleeding in hemophilia (18) We think that these examples demonstrate the usefulness of our concept of the hemostatic balance in the organism and its role in hemostasis tissue repair and connective tissue formation

Since the balance between fibrin deposition and fibrinolysis regulates wound healing and tissue repair this balance gains primary importance in the development of arteriosclerotic lesions (9) This concept is not necessarily contradictory to any of the lipid theories As a matter of fact lipids can become involved in this process in various ways The main difference is that we consider the hemostatic balance to be the process which governs tissue repair in the vessel wall and that participation of lipids in these processes is of secondary nature This does not exclude the possibility that the seriousness of the condition and the progression of the disease might depend partly on the effects of lipids

In order to discuss this a little more in detail we have to consider the anatomical structure of the arteries since the serious consequences of this process of tissue repair in the arteries depends on the special conditions prevailing there (9)

The high thromboplastic activity and the low fibrinolytic activity of the intimal tissue will tend to increase deposition of fibrin after injury as compared with other tissues where the balance is not shifted as much in this direction The circulating blood can supply much more fibrin to the injured surface than is needed for normal tissue repair The hemostatic balance in the circulating blood will therefore determine whether the deposition of fibrin at the site of injury will be excessive and how soon it will undergo resolution

If the fibrin layer stays too long it will become covered by the endo-

The deposition of fibrin on the interior of a vessel appears much more probable as the high latent activity of the coagulation process in blood would easily supply the necessary fibrin The deposition of fibrin on an arterial wall and its subsequent organization by fibroblasts migrating in from the intima therefore forms a normal part of tissue repair in the vessel wall Thus the fibrin layer has to be removed again almost as rapidly as it is deposited to prevent the formation of connective tissue This deposition of fibrin and its subsequent removal therefore depends on the balance between the coagulation process and the fibrinolytic system in blood

Duguid (21 23) believes arteriosclerotic lesions result from the formation of parietal thrombi and their subsequent transformation into connective tissue To substantiate this concept he has found that fibrin deposits on the arterial wall occur more frequently than assumed before (22) His observation has been partially confirmed by others (20)

In order to elucidate the role of tissues in the vessel wall in the deposition and resolution of fibrin we have studied the thromboplastic and fibrinolytic activities of the layers of the human aorta (14) Thromboplastic activities were compared with a suspension of human brain as used in the estimation of prothrombin by Owren s P P method We *found the preparations made from the tunica intima to be almost* as potent as the brain suspension Also the media was highly active though usually lower than the intima The adventitia showed only low thromboplastic activity The content of plasminogen activator in the layers was also estimated Here the adventitia had the highest activity while the media contained medium or low concentrations and the intima contained little or no activity This means that an intimal injury will release thromboplastic activity which can produce a deposit of fibrin on the intimal surface as the primary process of tissue repair The lacking fibrinolytic activity in the intimal tissue means that the fibrin cannot be removed again by fibrinolytic components produced by the tissue Its ultimate removal depends solely on the fibrinolytic agents in the circulating blood Again I believe Nature has had to compromise If the fibrin deposit could be dissolved by agents in the tissue then it could very easily be loosened from the vessel wall Tissue repair would be *hampered and the loosened fibrin could be carried away in the* blood stream and be of greater danger as an embolus This makes the balance between thromboplastic activity and fibrinolytic activity in the blood stream of great significance for the establishment of normal conditions in the organism and we are now trying to develop methods for estimating this balance

Arteriosclerotic lesions have recently been reported in hemophilic

probably not caused so much by their direct effects on the main vessels —although occlusion of these produces ischemia in the organ fed by the artery in question—but by the identical process occurring in the *vasa vasorum* of the artery and the injury produced by this effect on the vessel wall In this way it will probably be easier to explain an increase in the frequency of injuries in the arterial wall after prolonged partial vasoconstriction than by a direct effect The necrosis thereby produced can also release thromboplastic agents which can diffuse to

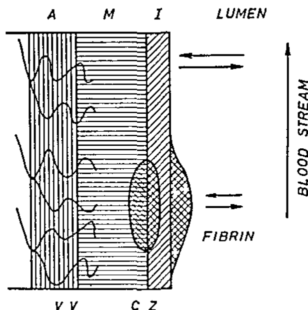


FIG 1 Diagram of arterial wall A = Adventitia (*tunica externa*) M = *Tunica media* I = *Tunica intima* The *vasa vasorum* in the adventitia and media is indicated as v v The diffusion is indicated by long arrows for normal diffusion and shorter for decreased diffusion The critical zone with decreased nutrition behind the fibrin deposit is marked c z

the lumen of the artery and give rise to fibrin formation and deposition I think this answers Dr Ambrus question about a possible role of the *vasa vasorum* in the pathogenesis of arteriosclerosis previously discussed by Winternitz (41) The concept is based on a metabolic disturbance in the avascular layers of the arterial wall leading to cell death and tissue necrosis Dr Hartroft describes it as "metabolic necrosis" The disturbance is produced by decrease of the diffusion of nutrients to the vessel wall either by deposition of fibrin or by interruption of the blood flow in parts of the *vasa vasorum* Because of the high thromboplastic activity the low fibrinolytic activity and the lack of

thelial cells and be separated from the blood stream Later it will undergo organization as described by Duguid (21) The significance of a rapid removal of excess fibrin from an injured intimal surface is immediately apparent since the removable at a later stage will be difficult because of the lacking fibrinolytic activity of the *tunica intima* Also no granulation tissue is formed as part of this wound healing be cause of the lack of capillaries in the central layers of the media and the intima The resulting process will produce a proliferation of fibro blasts in the intima followed by intimal thickening (21) This is normal tissue repair

However the formation of a fibrin deposit on an arterial surface not only represents the deposition of inert material but constitutes a barrier for the nutrition of the underlying layers of the wall The central layers of the wall in systemic arteries usually rely on the diffusion of oxygen and metabolic products to and from the blood stream for their nutrition This applies to the intima as well as to the central layers of the media The *vasa vasorum* which supply the adventitia and the ex ternal layers of the media do not extend far enough to feed the more central layers of the media and the intima Therefore if the fibrin barrier is not rapidly removed by fibrinolysis the cells in a critical zone behind the fibrin deposit will begin to die producing necrosis and in ducing degenerative processes This is schematically described in Fig 1

The necrotic process in the critical zone of decreased nutrition gives rise to degeneration It can release thromboplastin from the dying cells in the intima and media and thus increase fibrin deposition because of lack of fibrinolytic activity in these tissues The process in the arterial wall is more dangerous than in tissues rich in capillaries since the metabolic disturbances are much smaller in such tissues This applies also to the veins since the walls of veins are thinner and more highly vascularized than those of the arteries Furthermore venous blood has a higher fibrinolytic activity than arterial blood Quite recently Todd (39) has demonstrated by an ingenious micromodification of the fibrin plate method that the walls of veins are highly fibrinolytic There are thus several good reasons for a relatively lower frequency of phlebo sclerosis than of arteriosclerosis and the argument against Duguid's hypothesis that it does not explain the common absence of atheroma after thrombosis in veins seems untenable (30)

From the diagram in Fig 1 it is evident that a critical zone of de creased nutrition can also occur if the diffusion from the *vasa vasorum* into the central parts of the *tunica media* is prevented This can occur by occlusion of the small arteries in the *vasa vasorum* by small fibrin clots but also by a constriction of its vessels The effect of vasoconstrict ing compounds such as adrenaline in producing vascular lesions is

in oxygen pressure than in other tissues at the same time as the lactic acid production during anoxia can enhance cell death Since connective tissue cells can release large concentrations of alkaline phosphatase it is interesting that Moon has found that calcification begins in the *elastica interna* the connective tissue part of the media

Another effect of cell death in the critical zone is the release of growth promoting substances similar to those produced by cell injury in tissue cultures We assume this is the mechanism by which fibro blastic proliferation begins in the intima, and that in addition to the anoxic state it also constitutes the initial induction to growth of the capillaries from the deeper layers of the media to the more central layers in an effort to restore normal metabolism In this way the capil laries can move into the critical zone and become the site of subintimal hemorrhages

Duguid s concept as well as that presented here dates back to Karl von Rokitansky in Vienna Rokitansky describes many of the pathological processes in a similar way as set forth above although with other words I have recently discussed this interesting fact (12) and only a few specific points will be mentioned here

Rokitansky suggested that fibrinous deposits were produced on the vessel wall by an effect of the exudate released from injured tissue on some components in the blood This concept was one of the main reasons for Virchow s rejection of Rokitansky s idea since he thought blood clotting was caused by oxygen Intuitively Rokitansky reached views concerning physiological processes which forecast observations made many years later His concepts were developed before fibrinogen was known as an entity before thrombin was known before the clot promoting effect of the tissue extract was known and more than sixty years before its role as an activator and its difference from thrombin was revealed by Morawitz and Fuld and Spiro

In the understanding of the role of fibrinolysis on this process it is interesting to note that Rokitansky reports especially on the compara tively rare occurrence of arteriosclerotic lesions in the pulmonary arteries

This seemingly paradoxical observation is easily explained according to the concept presented here The pulmonary arteries carry venous blood which has a higher fibrinolytic activity than arterial blood The anatomical structure differs from that of the systemic arteries per mitting easier nutrition of the vessel wall Arteriosclerotic lesions would naturally develop less frequently in the pulmonary arteries than in the systemic arteries In the same way we can explain why tricuspidal stenosis occurs less frequently than mitral stenosis

Also uterine tissue contains large amounts of plasminogen activator

capillaries in the critical zone (see Fig 1) the process can easily spread to adjoining parts of the wall

Many problems in vascular pathology can apparently find their explanation in the balance between fibrin deposition and fibrinolysis One of the important problems in the pathogenesis of atherosclerosis is the role played by lipids One single mechanism would hardly suffice as an explanation of all pathological processes in a complicated organ like the vessel wall The role of lipids is considered to be chiefly of secondary nature although of special importance for the extension of the arteriosclerotic process It will suffice here to mention some of the ways in which lipids might be involved

1 By causing a shift in the hemostatic balance e g by increasing the activity of the latent clotting system in plasma or decreasing the activity of the fibrinolytic system

2 By being incorporated into the fibrin deposit during or immediately after its formation and thus decreasing further the diffusion of nutrients through the deposits

3 As a result of cell degeneration (fatty degeneration) in the critical zone

4 Derived from erythrocytes in minute hemorrhages [this source has especially been stressed by Hartroft (24)]

The hypothesis has been extended to include an explanation of the calcifying process in arteriosclerosis The deposits which appear in cultures of chicken heart fibroblasts when cultivated in the presence of excess organic phosphates consist of calcium phosphate released by the effect of an alkaline phosphatase on the organic phosphate (15) This alkaline phosphatase occurs in cells which undergo necrosis and signifies cell degeneration (25) While osteoblasts contain alkaline phosphatase as a sign of differentiation normal connective tissue fibroblasts contain no alkaline phosphatase but this enzyme is formed or released during cell degeneration

Thus we could explain the calcification in necrotized tissue as an effect of the alkaline phosphatase released during cell degeneration viz as a degenerative calcification This mechanism produces the calcification in tuberculous lymph nodes (26) We assume therefore that the calcification process occurring in arteriosclerosis is produced by alkaline phosphatase released from degenerating connective tissue cells Calcification should then occur in the critical zone of decreased metabolism described above (Fig 1)

This explains why calcification occurs in the deeper layers of the arteries as well as in the peripheral arteries since muscle cells in the media have a high oxygen consumption which produces a steeper fall

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Discussion

Dr Shainoff Dr Astrup's data seem to fit the hypothesis To explain the rapid turnover of fibrinogen it must be assumed that the disappearance of fibrinogen from plasma involves conversion to fibrin

A number of years ago Bailey Bettelheim Lorand and Middlebrook demonstrated that during conversion to fibrin peptides are split from fibrinogen by the action of thrombin Since the level of these peptides in plasma might provide an *in vivo* measure of the conversion we have developed a method for determination of these peptides from plasma with a 10% accuracy at levels of a few gamma per milliliter At present a measure of the rate of disappearance of the peptides from the circulating blood is needed There is good reason to believe the peptides will not disappear in much less than 6 hours

Dr Gitlin In an animal?

Dr. Shainoff Yes because it is an acidic peptide We use rabbits In addi

(4) This is especially true of the endometrial tissue (1) and this activity is hormonally regulated (3) The fibrinolytic activity of menstrual blood is caused by a plasminogen activator of the tissue type probably derived from the shed endometrium (2) Thus there is a theoretical basis for an increased fibrinolytic activity in the peripheral blood during menstruation as reported by some authors although lack of satisfactory estimation methods leaves the problem unsettled

However considerable significance can be related to this question since a cyclically repeated appearance of increased fibrinolytic activity in the blood might explain the lower frequency of arteriosclerosis in *fertile women as compared to males and to older women* *Heavy* physical work also produces fibrinolytic activity (28 29) This may account for the decreased frequency of arteriosclerosis in physically active people It *might even be the physiological basis for any beneficial* effects of exercise although the risk of an increased number of minute injuries must be considered

Furthermore the occurrence of the relation between serotonin and carcinoid lesions of the pulmonary arterial system (40) could possibly be explained as a diffusion of serotonin from the returning venous blood causing constriction of the supplying small arteries with interruption of normal metabolism in a critical zone of the vessel wall or endocardium followed by tissue necrosis fibrin formation and fibroblastic proliferation

For historical reasons it is interesting to recall the investigations by Ruffer in 1911 (34) on peripheral arteries of Egyptian mummies He finds a high degree of arteriosclerosis with extensive calcification This *shows that the disease is not solely a product of modern life However* Egyptian mummies probably represent only the higher classes who probably did less physical work than the rest of the population The disease perhaps has always been connected with civilized modes of life

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hibit clot lysis Then we added the lipid directly to the blood We found that it made no difference whether butter was fed or injected The three layers of melted butter (clear interface and cloudy layers) were all active

Then we used reconstituted milk solids presumably fat free and they were active too

Calcium was the only substance common to all these substances Adding small amounts of calcium in this system has the same effect of prolonging clot lysis We have done this using blood collected without anticoagulants in siliconized tubes and kept cold Therefore we believe that possibly calcium or some form of it may act as an inhibitor of fibrinolysis

DR ASTRUP We have not seen any significant effect from adding calcium to our test but if calcium is added in a fibrinolytic system before lysis is produced the clot will differ in behavior from the clot formed by thrombin alone There might be a difference in the velocity of the resolution of these two clots There are reports on this but we have not tried it

DR VON KAULLA By recording the formation of plasma clots with the thromb elastograph it can be demonstrated that clots formed in the presence of calcium have a much stronger tensile strength and firmness than clots formed by thrombin and if we have the same fibrinolytic activity under both circumstances the one formed with thrombin dissolves quicker than that with calcium

DR RATNOFF I think more than tensile strength is involved Fernleigh and Tweed (*Clin Sci* **12** 81 1953) showed that a clot formed from plasma on the addition of thrombin lyses more slowly in the presence of calcium than in its ab sence On the other hand if one precipitates out the crude euglobulin fraction which contains plasminogen and fibrinogen along with many other things one can form a clot upon the addition of thrombin which will then lyse (O D Ratnoff *J Exptl Med* **96** 319 1952) Paradoxically the addition of calcium ions will speed this lysis The intimate mechanism of the action of calcium ions therefore is still a little fuzzy

DR AMBRUS If you add streptokinase to your system you are actually measur ing plasminogen Did you increase the plasminogen level as you increased the measure of lysis activity in the animal?

DR HARTROFT We are doing a kind of screening work with this system using the formation of the clot and its lysis as the end point

DR AMBRUS But you took blood from the animal added streptokinase and observed lysis What kind of conclusion did you draw if lysis was accelerated?

DR HARTROFT Actually lysis was prolonged We simply concluded this was *in vitro* confirmation of our suspicion that the reason we see more lesions in the *in vivo* system is the high fat meals or whatever caused blood lysis was being interfered with

DR AMBRUS I think this can be interpreted the other way too If streptokinase is added to blood in a test tube it will convert any plasminogen in that tube to active plasmin Eventually a clot will form and later the plasmin will lyse this clot. If lysis time is prolonged it may indicate that the blood was deficient in plasminogen and thus no or little plasmin was formed under the effect of strepto-kinase An animal may have a low plasminogen level because its plasminogen is being converted *in vivo* to plasmin Plasmin is rapidly neutralized by the large excess of antiplasmin in the blood of most species and is often difficult to demon strate by simple methods

DR HARTROFT Purely in the *in vivo* setup the clots lasted longer

tion the studies of Christensen indicate that intravenously injected peptides have a half life of a few hours

DR GITLIN There are two problems here One concerns the equilibrium within the circulation represented by fibrinogen and the other deals with the equilibrium with respect to the site at which fibrin is deposited in the tissues Fibrinogen is catabolized primarily by the reticuloendothelial system Because it is turned over rapidly in the circulation indicates nothing about the hypothesis of whether fibrinogen is converted to fibrin in small amounts One can block most of the metabolism of fibrinogen by blocking the reticuloendothelial system

We have never been able to demonstrate fibrin as such in normal tissues by using the fluorescent antibody method or by labeling the fibrinogen with iodine washing the tissues and then looking for residual radioactivity If fibrinogen is converted to fibrin with subsequent fibrinolysis the amount of fibrin present at any one time is very small but the actual amount is relatively unimportant for your hypothesis

DR PAGE It is important to bring out the fact that the over all turnover may not reflect the local situation One should not use the total figure as a measure of what might occur locally Dr Gitlin s findings make us pause before too ready acceptance of the fibrin deposition concept yet I do not believe the evidence currently available negates it wholly It indicates that much more work is necessary before our views should become solidified

DR RATNOFF Dr Gitlin there is a congenital disorder afibrinogenemia in which the infant fails to synthesize fibrinogen in any detectable amount Strangely enough these patients survive because clotting is not the only mechanism by which the body prevents bleeding However some have died and I wonder whether they have anything within their blood vessels resembling atherosclerosis If they do then fibrinogen has no part in it if they do not perhaps it does

DR GITLIN There are two problems in this Children with afibrinogenemia are treated with fibrinogen as a rule Fortunately however bleeding is not a usual event in such children They may go along for many years without bleeding episodes and frequently fibrinogen has not been administered during this period of time

We had two children with afibrinogenemia who succumbed with bleeding episodes We found nothing resembling atherosclerosis However these were young children and too we did not have atherosclerosis in mind According to the hypothesis fibrin simply indicates the presence of damage to the vessel and while it may act as the nidus for a plaque it may not be essential for plaque formation

DR AMBRUS As the opposite of Dr Ratnoff's question there are a number of obstetrical accidents in which it was reported that fibrinogen is clotted out in large quantities coating the wall of the entire vascular system with fibrin deposits and producing a hemorrhagic disorder because of the absence of fibrinogen without fibrinolysis Many of these women survived Did they develop atherosclerosis?

DR RATNOFF For 8 or 9 years none of the women we have studied has died subsequent to recovery from the immediate effects of hemorrhage My hope is that I will survive some of them so I will find out what goes on

DR HARTROFT Dr Astrup our attention was directed to the lysis system by the fact that feeding high fat meals to rabbits injected with thrombi increased the incidence of persistent clots as organized lesions By simplifying this procedure and repeating these high fat meals then withdrawing blood and activating the lysis system we could show that meals of butter or oleomargarine would similarly in

The Fibrinolysin System Some Physiological Considerations*

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Ever since Dastre drew attention to fibrinolysis as an *in vivo* phenomenon physiologists have suspected that it may play a significant role in ridding the organism of intravascular and extravascular fibrin deposits Although interest and research in this field has been relatively limited and the knowledge which has accumulated is still superficial the problems of human disease have begun to focus increasing attention on this biological phenomenon For example some investigators have championed the view that deficient or impaired fibrinolytic mechanisms may be involved in the pathogenesis of atherosclerosis They propose that small thrombi are continually being formed in the vascular bed and then are cleared by normal fibrinolytic mechanisms When this balance is disturbed by impairment of the normal fibrinolytic mechanism then the thrombi instead of being dissolved become organized and form the site for the development of atheromatous plaques Although most investigators do not accept this relation between the persistence of thrombi and the pathogenesis of atherosclerosis a number of them believe that an impaired mechanism for the dissolution of thrombi exists in patients with degenerative vascular disease and contributes more to the development of vascular insufficiency than the coexisting atherosclerosis In addition these investigators suggest that the frequently associated alterations in serum lipids may be responsible for the impairment of the normal fibrinolytic mechanism Increasing interest in fibrinolysis as a therapeutic measure is also apparent The potential value of controlled fibrinolysis for the therapy of thromboembolic disease is obvious to all

The purpose of this presentation is to comment on some of the known aspects of the fibrinolysin system and to describe some of the more recent studies in our laboratories

Supported by grants from the National Heart Institute National Institutes of Health and Lederle Laboratories Division American Cyanamid Co

DR ALLEN We have been interested in Dr Astrup s experience in operating on patients with prothrombin deficiency This is not dangerous as long as the operating field is directly in view of the surgeon If one does even paravertebral block or paracentesis in the presence of marked prothrombin deficiency (around 20%) these patients may bleed fatally

We have had considerable experience with the treatment of cerebral thrombosis with anticoagulants In single episodes cerebral vascular insufficiency has no effect upon recovery but repeated ten or fifteen times a day heparin or dicumarol or one of its analogs stop the attacks immediately

Dr Astrup regarding your statement that clots are deposited on the arterial wall because the wall has a high thromboplastic activity while this is not so of intima of veins because of their low thromboplastic activity have you actually done any studies on this?

DR ASTRUP No we have not investigated the veins so far What I would suggest is that even if a deposit occurs on the veins it would be removed more rapidly than on an artery

DR ALLEN Dr Duguid have you studied veins to see if fibrin thrombi form on the intima of veins as well as on arteries?

DR DUGUID Dr Astrup s plate method has been modified by Dr Todd in my department by which he can show the histological distribution of fibrinolysin activators I have sections of his showing that veins are especially rich in fibrinolysin activators

DR ALLEN On study of the vena cava do you find deposition of thrombi such as you find in the aorta? Or have you made such a study?

DR DUGUID Yes They appear in the vena cava but much less commonly than in arteries Mural thrombi commonly occur in varicose veins but the thickenings which result are slight and seldom lead to occlusion

DR ALLEN Dr Astrup we see patients whom we call clotters and they are characterized by increased amounts of fibrinogen in the blood which is not very common or by a sharp acceleration in coagulation in the early phases of coagulation Do you know whether or not these patients are more susceptible to atheromatosis?

DR ASTRUP No we do not We are trying to develop methods for estimating quantitative differences in the balance between fibrin formation and fibrinolysis in the blood But I would expect these patients would have a higher frequency of atherosclerosis

DR ADLERSBERG Could you tell us what your concept or definition of meta bolic necrosis is?

DR ASTRUP My concept is that when there is decrease of the exchange of metabolic products from a vascular site tissue necrosis occurs This is caused by decrease in oxygen tension because fibrin deposits have prevented the diffusion of oxygen That is the basis of the necrosis in the arterial wall which can be produced by a fibrin deposit on the surface or by constriction or occlusion of the arteries in the vasa vasorum In both layers there will be an intermediate layer with a necrotic zone

DR ADLERSBERG In other words metabolic necrosis is caused by impaired oxygen uptake in the tissues secondary to impaired oxygen diffusion Is this what you mean?

DR ASTRUP Yes That is also where alkaline phosphatase appears because it is contained in dying cells

mum and the types of links it splits Additional plasma constituents which are susceptible to the action of plasmin include fibrinogen accelerator globulin and some of the components of complement

Since the action of plasmin is not confined to fibrin the terminology plasminogen and plasmin appears to be preferable to profibrinolysin and fibrinolysin and is in keeping with the terminology of trypsinogen and trypsin In this report reference will be made to plasmin and fibrinolysin interchangeably

II Characteristics of Plasminogen and Plasmin

In vivo plasminogen occurs in close relationship to fibrinogen and fibrin In the process of clotting, significant amounts of plasminogen are adsorbed onto the fibrin clot and in areas of exudation plasminogen appears simultaneously with fibrinogen and fibrin In addition studies of transudates and exudates reveal a good correlation between the concentrations of fibrinogen and plasminogen It has been impossible to prepare fibrinogen free of plasminogen We suspect that the close relationship between these two substances may be of considerable physiological significance However major separation of plasminogen from fibrinogen is simple since plasminogen has the characteristics of a globulin In the alcohol fractionation procedure of Cohn the bulk of the plasminogen is in Fraction III whereas the fibrinogen appears in Fraction I Recently Kline (8) has developed a simple method for the purification of plasminogen which consistently yields preparations which are 500–800 fold as purified as compared to serum By allowing these plasminogen preparations to activate spontaneously to completion in the presence of appropriate stabilizing agents plasmin preparations of comparable purity have been obtained (2) Although these plasminogen and plasmin preparations are still significantly contaminated with other proteins they have been proven to be homogeneous enough for physicochemical examination

Some of the preliminary physicochemical data obtained by Shulman *et al* (20) is summarized in Table I It will be noted that plasminogen is a fairly large protein with a molecular weight estimated at 143 000 The viscosity data suggests that it has a 8 1 length to thickness ratio About 25% of the protein has been demonstrated to be split off in the conversion of plasminogen to plasmin This observation is in fair agreement with physicochemical data which estimates a molecular size for plasmin of approximately 108 000 There is approximately 1% carbohydrate in both plasminogen and plasmin suggesting that these globulins may be glycoproteins

I COMPONENTS OF THE HUMAN FIBRINOLYTIC ENZYME SYSTEM

The known components of the human fibrinolytic enzyme system are shown in Fig 1

The naturally occurring precursor of the proteolytic and fibrinolytic enzyme of serum is referred to as plasminogen although the term profibrinolysin remains a favorite among many coagulationists In the presence of a kinase or activator this normal serum globulin is converted to plasmin or fibrinolysin Serum tissue and urinary kinases have been described but streptokinase has been used most widely for studies on the system under consideration Although streptokinase was believed to activate plasminogen directly recent work suggests that it

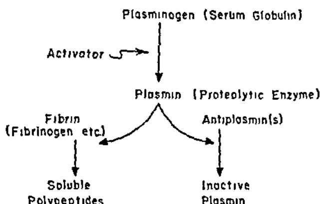


FIG 1 Components of the human fibrinolytic enzyme system

first reacts with a serum factor to form an activator (13 22) Although evidence is accumulating that a proactivator is present in plasma (5) sufficient isolation and characterization required to prove the existence of this substance has not been accomplished Free plasmin in blood is rapidly inhibited by one or more antiplasmins found in serum and platelets When the antiplasmin activity is removed by chloroform acetone or other similar physical agents slow but progressive spontaneous activation of the plasminogen occurs Although antiplasmin activity undoubtedly plays a significant role in controlling the fibrinolytic enzyme system we know very little about these inhibitors at the present time Accumulation of knowledge in this latter area undoubtedly will lead to a better understanding of the plasminogen plasmin system

Plasmin although it digests fibrin into several soluble incoagulable fragments is not restricted in its action to fibrin It is a proteolytic enzyme resembling trypsin in many respects particularly in its pH opti

Smyrniotis (21) in our laboratories has studied the daily urokinase excretion of patients with a variety of diseases. Significant reduction in urokinase excretion occurred in patients with renal insufficiency, advanced hepatic cirrhosis, and in some malignancies. Surprisingly, an abnormally high excretion occurred in patients with myocardial infarction. Of considerable interest is that the increase was not evident at the time of infarction but appeared during the second week and persisted for several weeks thereafter, suggesting that the urokinase excretion may reflect events of considerable physiological significance. Streptokinase, a bacterial product produced by hemolytic streptococci, has been previously referred to as a potent plasminogen activator. Similarly, the activator staphylokinase may be obtained from hemolytic staphylococci. Finally, trypsin is capable of activating plasminogen.

Recently we have completed kinetic studies on the spontaneous activation of plasminogen (2), as well as by streptokinase, trypsin, and urokinase (3). In all of the studies there appeared to be a common denominator, namely, that the activation involved an enzymatic proteolytic step. All of the activations were associated with a release of 25–30% of the plasminogen nitrogen, and in each instance the activation appeared to be associated with a proteolytic enzyme capable of hydrolyzing arginine and lysine esters. One may suspect then that the tissue kinases or the serum kinase which appears in blood under certain circumstances are proteolytic enzymes with a special affinity for plasminogen.

IV. Physiological State of Fibrinolytic Activity

Until recently we suspected that the fibrinolytic system was a relatively inert one, becoming active only under very stressful conditions such as severe shocking episodes, amniotic fluid emboli, or extensive hepatic necrosis. However, recent studies indicate that the opposite is true—the fibrinolytic system is indeed dynamic and changes in activity are continually occurring. These changes in activity are mediated by the release of activator into the circulation.

Figure 2 cites the results of studies of fibrinolytic activity in the blood of patients following electroshock, pyrogen injection, and local ischemia. Increased fibrinolytic activity had been reported previously as occurring following each of these procedures. The fibrinolytic activity was studied by estimations of whole blood clot lysis time, the plasma dilution technique of Macfarlane (11) as modified by Fearnly (7), the fibrin plate method of Astrup (4), the lysis time of a clot formed from the plasma euglobulin fraction (12), and the ability of the plasma euglobulin to lyse preformed I^{131} tagged human plasma clots (17). The

TABLE I
PHYSICOCHEMICAL STUDIES ON HUMAN PLASMINOGEN AND PLASMIN (20)

Property	Plasminogen	Plasmin
Sedimentation constant	4 28	3 56
Diffusion constant ($cm^2\ sec^{-1}$)	$2\ 92 \times 10^{-7}$	—
Molecular weight	143 000	108 000
Intrinsic viscosity	0 08	—
Absorption spectrum (maximum)	2800 A	2800 A
Tyrosine	5 91	6 32
Tryptophan	3 78	4 04
Electrophoretic mobility (pH 2 1)	8 2 and $7\ 3 \times 10^{-5}$	$8\ 9 \times 10^{-5}$
Isoelectric point	5 6	6 2
Hexose %	0 98	1 51

III ACTIVATION OF PLASMINOGEN

Since fibrinolysis is dependent on plasmin but the naturally occurring substance is plasminogen much interest is attached to the mechanisms by which plasmin may be formed Shown in Table II are the two known major mechanisms by which plasminogen may be activated either spontaneously (when inhibitors are lacking) or by specific activators or kinases As a result of Astrup s studies (5) we now recognize that almost all tissues contain fibrinolysin activators Astrup believes that the differences in concentration of activators among the various tissues may prove to have important physiological significance There is little doubt that a fibrinolysin activator may appear in the serum under certain circumstances Urokinase is an activator present in human urine Whether it represents excreted tissue activator or is locally secreted by the excretory epithelium is unknown However it has been extensively purified by Ploug in Denmark (14) and we have obtained preparations for study which are about one twentieth as active as our most highly purified streptokinase preparations

TABLE II
ACTIVATION OF PLASMINOGEN

1 Spontaneous
2 Activators
   A Tissue activators
   B Serum activator
   C Urokinase
   D Streptokinase
   E Staphylokinase
   F Trypsin

each case it was possible to demonstrate the presence of a plasminogen activator as well as an enhanced ability of the plasma euglobulin to lyse labeled preformed human plasma clots

The presence of a plasminogen activator in the plasma of these patients was demonstrated by activation studies in which the plasma euglobulin was employed as the source of activator and purified human plasminogen as the substrate The amount of plasmin activated was measured by a fibrinolytic assay The sensitivity of this technique to trace amounts of the activator urokinase as well as some of the observations on patients are shown in Fig 3

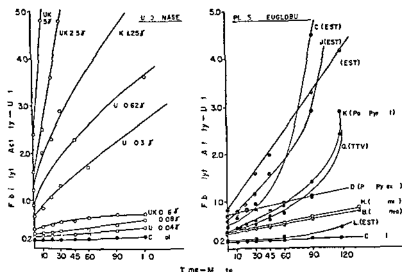


Fig 3 Activation of human plasminogen by urokinase and plasma euglobulin of patients (post EST pyrogens and ischemia)

On the left of Fig 3 is shown the amount of plasmin in fibrinolytic units appearing during the course of a 2 hour incubation at room temperature in a purified human plasminogen preparation containing varying amounts of urokinase When purified preparations of spontaneously activated human plasmin were added to the plasminogen preparation instead of activator no increase in fibrinolytic activity was noted during the entire period of observation On the right of Fig 3 are observations made with the plasma euglobulins derived from patients who developed enhanced fibrinolytic activity following electroshock pyrogens or ischemia Note that, in each instance shown the presence of the plasma euglobulin resulted in activation of the plasmin

plasma euglobulin techniques proved to be most sensitive probably due to the removal of inhibitors in isolating the euglobulin Shown in Fig 2 are the results of the lysis time of clots formed from the isolated plasma euglobulin and expressed in arbitrary fibrinolytic units with one unit of activity representing a lysis time of 30 minutes

Note, on the left of Fig 2 that the plasma euglobulin of almost all of the 55 patients who received electroshock therapy revealed an increased fibrinolytic activity 1 minute following electroshock In the pyrogen group shown in the center panel which consisted of 4 pyrexal treated patients and 1 triple typhoid vaccine treated patient enhanced fibrinolytic activity appeared shortly after but not before the chill

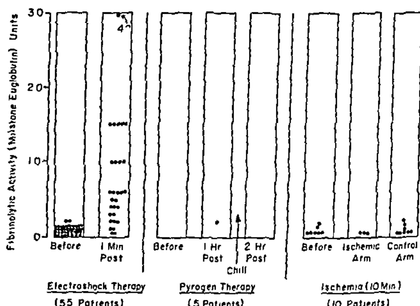


FIG 2 Fibrinolytic activity of plasma euglobulin in patients following electro shock therapy pyrogens and ischemia

Finally, as shown on the right of Fig 2 several of the patients subjected to a 10-minute period of local ischemia by the Kwaan technique (10), showed enhanced fibrinolytic activity in the blood drawn from the ischemic arm as contrasted to the preischemic level or to the opposite control arm

Studies on the plasma of those patients who developed the most intense fibrinolytic activity failed to reveal any evidence of free plasmin in the plasma or plasma euglobulin or alterations in the plasma fibrinogen plasminogen, or antiplasmin concentrations (18) However in

At present it is believed generally as shown on the left of Fig 4 that clot lysis results from the direct action of circulating fibrinolysin on the fibrin meshwork of a clot With this mechanism the concentration of circulating fibrinolysin or plasmin is critical in determining the rate of fibrinolysis Our own studies suggest an entirely different mechanism for thrombus lysis (1 17) Under this scheme as shown on the right of Fig 4 clot lysis results from the diffusion of activator into the thrombus with resultant activation of the intrinsic profibrinolysin or plasminogen followed by lysis of the clot With this mechanism the level of activator in the circulation rather than the level of circulating plasmin becomes the critical factor in controlling the rate of fibrinolysis These considerations which are of considerable physiological significance also are crucial for determining an approach to fibrinolytic therapy in man i e whether to concentrate on maintaining a high level of circulating plasmin or a high level of circulating activator

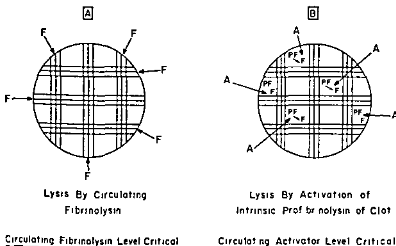


FIG 4 Schemes of clot lysis

1 *Evidence Against Circulating Plasmin Hypothesis*

Before presenting some of the evidence which has led us to suggest that the scheme on the right of Fig 4 may be an important physiological mechanism for thrombus dissolution let us consider why it is unlikely that thrombi are dissolved by circulating plasmin Our major objections are that the circulating plasmin mechanism does not take into consideration the important aspect of plasma inhibition it does not explain the special fibrinolytic properties seen with this enzyme system and

ogen In some cases the activation curves are of a different shape than that seen with urokinase a point which requires further investigation Specimens from these patients taken prior to the appearance of enhanced fibrinolytic activity did not activate plasminogen significantly

The appearance of enhanced fibrinolytic activity in these patient studies was correlated with the appearance of a plasminogen activator in the circulation but without significant change in any of the other components of the plasma fibrinolytic enzyme system Results similar to these also have been obtained following intense exercise or the administration of epinephrine

These experiments have demonstrated that activator may be released in or into the circulation under a variety of conditions and has stimulated our thinking along several lines

First we can observe trace amounts of fibrinolytic activity in the plasma of all patients and this activity varies considerably from patient to patient It may be questioned whether this observation is an artifact of the test system since almost all of the plasma inhibition is removed by the euglobulin precipitation prior to assay Recently we have been developing highly sensitive techniques for measuring activator activity and the preliminary results suggest that there is indeed free activator present in normal plasma As soon as these methods have been properly standardized a study of the levels of activator as they relate to age sex disease etc is planned

Second the electroshock experiments in which activator appeared within a minute suggest that a neurogenic mechanism may control the release of activator Of course electroshock is associated with convulsive movements which may contribute to the appearance of fibrinolytic activity however we have been able to block the convulsive movements with succinylcholine and still obtain evidence of activator release

Third the response to local ischemia which is limited primarily to the ischemic arm indicates that the activator is locally released as originally suggested by Kwaan and associates (9) The site of origin of the activator is unknown but we suspect that it may be derived from the connective tissues or perhaps from the endothelium of the vascular bed An intriguing thought is the possibility that individuals may vary in their ability to release activator in response to various stresses If this is true than there may be an explanation for the "spontaneous" dissolution of certain thrombi and not others

V Mechanism of Lysis of Thrombi

The significance of activator in physiological fibrinolysis becomes more apparent when one considers the mechanism by which thrombi are actually lysed

a somewhat longer period However here again significant plasmin and fibrinolytic activity disappeared shortly after plasminogen activation was completed

On the right of Fig 5 at the lowest SK concentration the activation occurred at the slowest rate and here significant amounts of plasmin and fibrinolytic activity persisted throughout the experimental period

Analysis of this data suggests that in the presence of normal plasma inhibition previously activated plasmin and fibrinolytic activity rapidly disappear The persistence of plasmin and fibrinolytic activity is associated with the continuing activation of new increments of plasminogen rather than being due to the presence of previously activated enzyme

Observations of this type as well as direct measurements of the inhibitory power of plasma upon plasmin indicate that in the presence of normal amounts of plasma inhibition it would be very difficult to maintain significant levels of circulating plasmin unless there were significant amounts of activator present at the same time

We also have been concerned that the circulating plasmin hypothesis does not explain the special fibrinolytic properties seen with this enzyme system As has been repeatedly demonstrated, plasmin is not restricted in its action to fibrin In purified solutions plasmin will digest a number of proteins and its actions upon fibrinogen and fibrin proceed at approximately equal rates (6) Some of the plasma components which are digested by plasmin include fibrinogen Ac globulin and some of the components of complement If the body were to rid itself of thrombi by the circulating plasmin hypothesis then the appearance of fibrinolytic activity in the blood should be associated with multiple hemostatic defects involving fibrinogen Ac globulin and perhaps others as well However blood removed from patients following electroshock or pyrogen therapy show striking fibrinolytic activity although fibrinogen levels are unaltered This special fibrinolytic activity i e greater fibrinolytic activity than fibrinogenolytic or evidence of other proteolytic activity is observed only in the presence of activators

Table III shows data on fibrinolytic activity as compared to fibrino-

TABLE III
FIBRINOLYSIS VERSUS FIBRINOGENOLYSIS (15)

Reagents	Fibrinolysis time in minutes	Fibrinogenolysis time in minutes
Human plasmin + human plasma	5	5
Streptokinase + human plasma	4	>60
Urokinase + human plasma	5	>60

it is not consistent with our *in vivo* observations on the lysis of fibrin deposits in experimental animals

The extent of the inhibitory activity of plasma upon plasmin may be demonstrated by the data shown in Fig 5 The data are derived from an experiment in which aliquots of normal human plasma were incubated with various concentrations of streptokinase at 37°C At intervals during the incubation period specimens were removed for assays of plasminogen plasmin and the plasma clot lysis time Plasminogen and plasmin activity were measured by casein proteolysis The top half

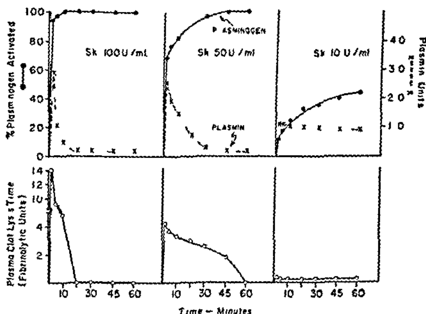


Fig 5 Activation of plasminogen plasmin concentration and plasma clot lysis time during incubation of plasma at different Sk concentrations

of Fig. 5 cites the percentage of the available plasminogen activated (shown by the solid circles) and the plasmin activity (shown by the crosses) In the lower half of the graph is shown the lysis time of plasma specimens clotted with thrombin expressed in fibrinolytic units

Note on the left of Fig 5 that at a streptokinase concentration of 100 units per milliliter all the plasminogen was rapidly activated the plasmin concentration rose sharply and then quickly fell to trace levels Similar changes were noted in the fibrinolytic activity of the plasma

In the center panel of Fig 5 where the activation proceeded at a somewhat slower rate plasmin and fibrinolytic activity persisted for

quantity to sustain significant levels of circulating proteolytic activity prothrombin and fibrinogen concentrations were reduced to zero levels and the blood rendered incoagulable Despite these drastic biochemical changes there was no increase over the control rate in the number of clots dissolved No observations were made of the effect of intravenous trypsin on the experimental peritoneal exudate

In the center panel are shown the effects of an injection of a mixture of streptokinase and human plasminogen This mixture not only contains human plasmin but also provides an excellent activator for dog plasminogen (19) and probably removes antiplasmin activity as well

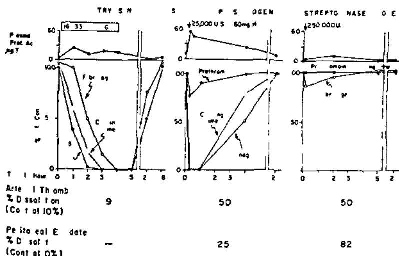


FIG 6 The effect of intravenously administered trypsin streptokinase plus human plasminogen or streptokinase alone in dogs with either an experimental femoral artery thrombus or a traumatic fibrinous peritonitis

Immediately following the injection complete activation of the dog plasminogen occurred and there was a tremendous burst of proteolytic activity The level of proteolytic activity then progressively returned to the control Associated with this burst of proteolytic activity the blood was rendered incoagulable as a result of the disappearance of circulating fibrinogen The one stage prothrombin time was transiently lengthened primarily as a consequence of Ac globulin depletion With this type of therapy it was observed that in 50% of the animals the arterial thrombus was lysed and in 25% of the animals the peritoneal exudate was completely dissolved

In the panel on the right of Fig 6 are the observations made with

genolytic activity in human plasma to which either human plasmin streptokinase or urokinase has been added For this experiment normal human plasma was incubated at 37°C To one specimen a sample of spontaneously activated human plasmin was added At zero time an aliquot was removed clotted with thrombin and the lysis time of the clot determined This is referred to as the fibrinolysis time At frequent intervals thereafter additional aliquots were removed and the time determined when no clot would form upon the addition of thrombin This is referred to as the fibrinogenolysis time To a companion specimen of human plasma streptokinase or urokinase instead of human plasmin was added and similar measurements were made It will be noted in Table III that the addition of human plasmin in sufficient concentration produced a disappearance of fibrin and fibrinogen at the same time In *contrast the addition of streptokinase to plasma produced rapid* fibrinolysis but significant amounts of fibrinogen still remained after 60 minutes Similar findings were observed when a purified urokinase preparation was added to plasma Thus the special fibrinolytic activity as compared to fibrinogenolytic activity appears to be associated with plasminogen activators rather than plasmin itself Since we were able to demonstrate that plasminogen was being continuously activated during the period of incubation with the streptokinase or urokinase our data suggests the following explanation for this special fibrinolytic activity (1) plasmin arising from the activation of plasminogen in plasma *is rapidly inhibited by antiplasmin resulting in protection* of the fibrinogen and other plasma components (2) when clotting occurs the plasmin arising from the activation of plasminogen in the clot produces rapid fibrinolysis

Additional evidence which casts doubt upon the circulating plasmin hypothesis may be obtained from an analysis of our previous *in vivo* observations on the enzymatic dissolution in animals of experimentally induced *thrombi (19)* and fibrinous *exudates (16) In these experi*ments two groups of dogs were studied In one group femoral artery thrombi were produced while in another group a traumatic fibrinous peritonitis was induced The animals were treated intravenously either with trypsin human plasminogen activated with streptokinase or streptokinase alone

Some of the results of these observations are cited in Fig 6 The top half of Fig 6 refers to some of the biochemical changes observed with each of the agents used while the lower half refers to the effects on the thrombi or fibrinous exudate in terms of the percentage of animals in which the clot or peritoneal exudate was completely dissolved On the left of Fig 6 note that when trypsin was given in sufficient

proteolytic activity produces marked clot lysis an effect directly related to the streptokinase concentration On the right of Fig 7 it will be noted that the addition of streptokinase to the previously ineffective plasmin preparation does not increase the proteolytic activity of the mixture but results in a powerful clot lysing preparation The enhanced activity observed with streptokinase and plasmin may be due to the formation of increased amounts of activator

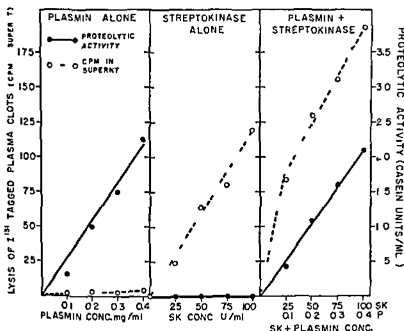


Fig 7 Effect of plasmin and streptokinase on I^{131} tagged human plasma clots Reprinted from Sherry *et al* (17)

From these and similar observations utilizing other activators (e g urokinase) as well as other techniques (e g studies on the rates of lysis of plasminogen rich and plasminogen deficient clots) we have concluded that the ability of plasma to lyse thrombi is dependent upon its activator content but relatively independent of its proteolytic activity or plasmin content

Additional evidence favoring the circulating activator view has been obtained from patient studies where we are attempting to produce controlled fibrinolysis by maintaining high levels of circulating activator

Figure 8 illustrates the biochemical changes induced on the second day of treatment in an 84 year old man suffering from a femoral artery

large injections of streptokinase alone With this type of injection the plasma inhibition remained unaffected and there was very little evidence of any increase in circulating proteolytic activity No changes were observed in the prothrombin or clotting time and only a minor fall in the fibrinogen concentration occurred However blood removed shortly after the injection showed whole blood fibrinolysis and there was a moderate fall in plasminogen concentration Despite these minor biochemical changes as contrasted to the center panel 50% of the arterial thrombi were also dissolved completely and the remaining clots were softer and smaller than the other groups With this type of therapy the peritoneal exudate was lysed in 82% of the animals

Thus in these animal experiments success in dissolving preformed fibrin *in vivo* did not appear to depend on the level of circulating *plasmin or proteolytic activity* indicating that other factors were probably of greater importance

2 *Evidence for Activation of Intrinsic Clot Plasminogen*

Let us consider the evidence to support the second scheme (shown in Fig 4) that clot lysis may result from the diffusion of activator into the clot since all thrombi contain intrinsic plasminogen If this scheme were operative then the level of circulating activator becomes the critical factor in determining the rate of fibrinolysis of preformed thrombi

Figure 7 cites sample data obtained from in vitro studies which indicate that the rate of lysis of preformed *human* plasma clots is much more sensitive to the presence of surrounding activator than to plasmin The rate of fibrinolysis was measured by the amount of radioactivity released from preformed I^{131} trace labeled human plasma clots The clots were incubated in solutions containing either human plasmin alone activators alone or mixtures of plasmin and activator The proteolytic activity of the test solutions were measured by a standard casein *method The data in Fig 7 are presented in three panels* on the left are shown the effects of a purified preparation of human plasmin alone in the center the effects of streptokinase alone and on the right the results obtained with a mixture of streptokinase plus plasmin The broken line refers to radioactivity released from the tagged plasma clots while the solid line refers to the proteolytic activity of the substance under investigation The data in each panel are plotted as a function of the concentration of the substance under study

It will be noted on the left of Fig 7 that plasmin at very significant levels of proteolytic activity produces very little lysis of the tagged clots In sharp contrast as seen in the center streptokinase with no

the absence of significant proteolytic activity in the plasma showed a virtual absence of *plasmin* in the circulation This finding was confirmed by the presence of a sustained fibrinogen In contrast as shown in the lower graph the streptokinase injections were associated with a striking rise in the clot lysing activity of the plasma which was maintained during the period of SK injections but returned to the pretreatment range by the end of the period of observation These data illustrate in an unequivocal fashion the dissociation of the *thrombolytic power of the plasma* i e the ability of the plasma to lyse a thrombus from its plasmin content

We believe that these data provide fairly strong evidence that the concentration of circulating activator surrounding a thrombus is more critical than the level of plasmin in effecting the lysis of preformed thrombi suggesting that activation of the intrinsic clot plasminogen is an important mechanism for the lysis of preformed fibrin

To test the validity of this view an investigation was undertaken of the fibrinolytic activity which appears in man following electroshock, pyrogens ischemia exercise and epinephrine As previously noted the appearance of enhanced fibrinolytic activity in these patient studies was correlated with the appearance of a plasminogen activator in the circulation as well as an increased ability to lyse preformed human plasma clots but without significant change in any of the other components of the plasma fibrinolytic enzyme system These observations in stress situations where the body responds with enhanced fibrinolytic activity are entirely consistent with the activator hypothesis

VI Concept of Physiological Mechanism for Fibrinolysis

Figure 9 summarizes our present concept concerning the physiological mechanism for fibrinolysis Under this scheme the body responds to certain stimuli by releasing activator in or into the circulation In plasma the strong inhibitory power of the plasma will rapidly inactivate any plasmin formed protecting the fibrinogen and other susceptible protein components from the action of this proteolytic enzyme The diffusion of activator into any fibrin containing exudate will activate the intrinsic plasminogen in a relatively inhibitor free area and cause lysis of the fibrin By this mechanism the fibrinolytic enzyme system is endowed with special fibrinolytic properties which plasmin itself does not possess One may also note that under this hypothesis the importance of plasminogen in the circulation is not to induce fibrinolysis but to endow thrombi or fibrinous exudate with sufficient plasminogen to mediate its subsequent lysis Finally if our concept is correct then the emphasis in the developmental work for producing controlled fibrinolysis in man

thrombosis The patient received three 200 000 unit injections of highly purified streptokinase at 2 hour intervals and observations were made over a 16 hour period The top graph describes the plasminogen and fibrinogen levels Immediately beneath the top graph are observations on the lysis time of spontaneously clotting whole blood specimens In the lower graph are shown observations on the ability of the patient's plasma to produce zones of lysis on the fibrin plate test and to lyse radioactive human plasma clots

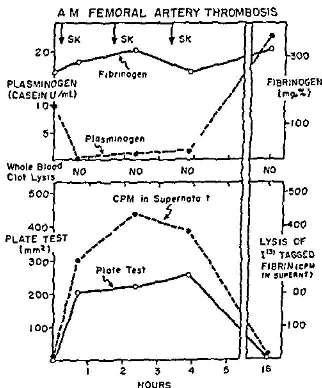


FIG 8 Biochemical change induced by intravenous streptokinase in a patient with a femoral artery thrombosis Reprinted from Sherry *et al* (17)

It will be noted that the fibrinogen levels were not significantly altered during the entire period of observation Immediately following the first streptokinase injection the plasminogen concentration fell to insignificant levels as a consequence of its rapid activation and remained depressed during the period of repeated injections At the end of the period of observation the plasminogen concentration had returned to a high level None of the spontaneously clotted whole blood specimens lysed over a 24 hour period and this observation together with

Components of the Human Fibrinolytic System in Blood and Urine and Their Relationship to Therapeutic and Spontaneous Fibrinolysis

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A sterile blood clot incubated in its own serum will dissolve This phenomenon may in normal blood require many days In pathological blood it may occur in a few minutes This dissolution also known as the fourth phase of coagulation is commonly referred to as fibrinolysis It results from the adsorption by fibrin of fibrinolytic enzymes from the blood Because of this the pioneers in fibrinolysis research were able to obtain fibrinolytic enzymes by extracting washed fibrin with acetic acid (8) The fate of the clot *in vivo* i e the intravascular clot or any fibrin deposit in the tissues is basically determined by two independent processes organization or enzymatic disintegration The fibrinolytic disintegration of the fibrin network was observed microscopically by Fonio (5) to parallel the appearance of a "rubber like" mass which subsequently disappeared by itself This shows that there is an inter mediate phase of clot dissolution which is a challenge for further in vestigation A definite predominance of enzymatic breakdown over or ganization may result in complete dissolution of the clot whereas a predominant organization prevents enzymatic dissolution because an or ganized clot or fibrin strand is no longer accessible to fibrinolytic en zymes Recently histological evidence has been presented indicating that thrombi frequently undergo partial lysis before being organized (9) During the clinical course of a thrombosis an increase in the fibrinolytic activity of the circulating blood can be demonstrated (11 12) It seems desirable in the attempts to eliminate or prevent pathologi cal fibrin deposit the human organism should be assisted in activating its own endogenous fibrinolytic potentialities For this purpose it seems a more logical approach to utilize endogenous human enzyme sources than to administer exogenous enzymatic material which has been shown to be antigenic

The human fibrinolytic potential is very great as demonstrated in Fig 1 These data show the development of both fibrinolysis activity

should be on inducing and maintaining sustained levels of circulating activator rather than circulating plasmin This approach has been emphasized in our patient studies

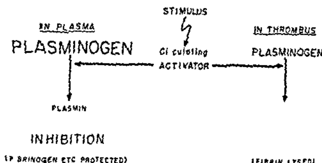


FIG 9 Concept of physiological mechanism for fibrinolysis (normal plasma inhibition)

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See DISCUSSION (page 279)

and of a circulating anticoagulant following open heart surgery in a girl The histograms of the upper row pertain to the circulating anticoagulant and the histograms of the lower row pertain to fibrinolysis The histogram set B of the lower row shows that addition of 20% of the patients plasma to normal plasma induced strong fibrinolysis of the normal clotted plasma within 25 minutes This indicates that this particular patient (at the time of the test) had activated to plasmin approximately four times as much of her plasminogen as would have been required to dissolve her clots within a few minutes

Attempts to activate the human potential fibrinolysis for therapeutic purposes are not new These attempts have been hampered by two basic deficiencies first the scanty knowledge of the mechanism of appearance of spontaneous fibrinolysis in man and second the variety of methods used for estimation of this phenomenon or of separate components taking part in it In the study of fibrinolysin information gained from the use of the bacterial activator streptokinase has further confused the picture because the addition of streptokinase to human blood activates proteolytic enzymes which behave differently from the enzymes obtained by activating human blood with a human activator (3 6) We are able to demonstrate in Table I that human plasma in which fibrinolysis is induced by streptokinase breaks down synthetic amino acid esters whereas plasma in which a comparable intensity of fibrinolysis is induced by the human activator urokinase does not (10) This would indicate that in the presence of naturally occurring inhibitors in the plasma the plasmin brought about by streptokinase activation has properties that are different from that obtained by urokinase activation

TABLE I

THE EFFECT OF UROKINASE ON SPONTANEOUS AND STREPTOKINASE ACTIVATED ESTERASE ACTIVITY IN PLASMA

Condition	Tosyl arginine	Lysine	Lysis time
H_2O (13)[b]	0 180 ± 022	0 018 ± 005	—
Strep (13)	0 175 ± 029	0 020 ± 007	—
Uro (4)	0 210 ± 075	0	—
Uro + strep (4)	0 172 ± 021	0	—
Plasma (12)	0 206 ± 020	0 022 ± 006	>24 hr
Plasma + uro (12)	0 206 ± 046	0 023 ± 007	20–50 min
Plasma + strep (12)	0 580 ± 029	0 405 ± 040	20–60 min
Plasma + uro + strep (12)	0 540 ± 005	0 292 ± 030	30–60 min

Expressed as mean increase in μ moles of acid liberated per 60 minutes incubation From von Kaulla and Schultz (17)

[b] Numbers in parentheses indicate number of determinations

Mean ± standard error

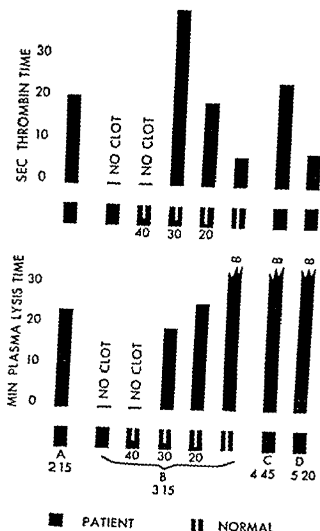


Fig 1 Trend of circulating anticoagulant plus marked fibrinolysis activity developing during open heart surgery with pump oxygenator Peak activity 2 hours after protaminization Serial determinations of thrombin time and coordinated plasma lysis time

Upper set of histograms Thrombin time Histogram A prolonged thrombin time of patient s plasma Histogram B shows in succession the following incoagulability of patient s plasma 40% of which makes normal plasma incoagulable 30% and 20% prolong thrombin time of normal plasma considerably and thrombin time of normal plasma Histogram C and D anticoagulant activity subsides Lower set of histograms Plasma fibrinolysis Histogram A, plasma lysis in 23 minutes Histogram B shows in succession the following incoagulability of patient s blood 40% mixture of patient s with normal 30% and 20% of patient s plasma induce rapid lysis in normal plasma which itself does not lyse within 24 hours and normal plasma without lysis Histograms C and D *no more fibrinolytic action demonstrable* Note time scale From von Kaulla and Swan (18)

ticular type of fibrinolysis The thrombelastograms or coagulograms show the appearance of a circulating anticoagulant (coagulogram B) and spontaneous fibrinolysis followed by paracoagulation (coagulogram F) Paracoagulation may be tentatively explained as an incomplete formation of the initial clot which then undergoes fibrinolysis During this process thrombin is adsorbed on the fibrin but is released with

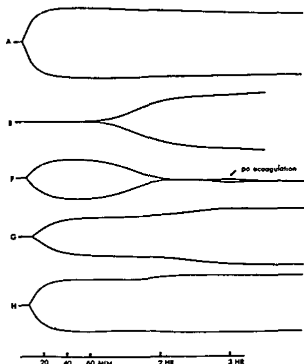


FIG 3 Serial coagulograms from citrated recalcified plasma taken at intervals during open heart surgery A control before anesthesia B after anaesthesia during opening of chest Considerable delay in beginning of fibrin formation due to circulating anticoagulant F after termination of by pass beginning of normal fibrin formation followed by clot lysis within 2 hours Paracoagulation G and H obtained 30 and 60 minutes later respectively show absence of fibrinolysis Note time scale From von Kaulla and Swan (18)

fibrinolysis and clots the remaining fibrin which again may redissolve

The second technique is the observation of spontaneous fibrinolysis of the isolated euglobulin fraction Normal euglobulins when redissolved and clotted always undergo spontaneous fibrinolysis The euglobulins are precipitated by diluting the plasma and saturating the solution with CO_2 Clotting is effected by adding thrombin to the euglob

For this and other reasons human components were used exclusively in our fibrinolysis studies The human activator of plasminogen uro kinase was obtained from human urine by a procedure shown in Fig 2 Incidentally with the same procedure a powerful thromboplastic material is obtained from the urine seen on the left side of the chart This material was sterilized and was very helpful for local treatment of any bleeding due to deficiencies in thromboplastin formation (13)

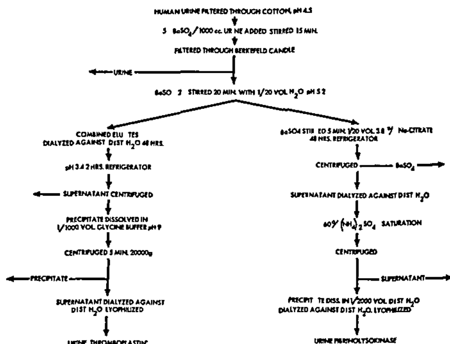


Fig 2 Flow diagram with the essential steps for the preparation of urokinase and urothromboplastin from human urine

In measuring the fibrinolytic tendency of the blood two techniques were used The first was thrombelastography which permitted a con tinuous automatic recording of fibrin formation and fibrin dissolution and gives insight into the kinetics of the dissolution (14) Thromb elastography also enabled us to observe phenomena such as paracoagu lation which is the reformation of a clot after its complete dissolution As mentioned it was found that marked fibrinolysis tendency may de velop during open heart surgery with by pass procedure particularly if the perfusion rate is low Figure 3 reproduces serial coagulograms obtained from such a patient at intervals during surgery as representa tive examples of the characteristics of thrombelastography and this par

euglobulin lysis times (indicated by an arrow pointing downward) was obtained from another patient undergoing heart surgery with the by-pass procedure. Note time scale and the progressive shortening of the euglobulin lysis time indicating increasing lysis tendency not yet reflected by the coagulograms. Coagulogram C is a straight line because no clot is formed due to heparinization. The corresponding euglobulin lysis time reveals a considerably increased lysis tendency. Coagulogram D again demonstrates the advantage of the euglobulin lysis time in providing quick answers. The euglobulin fraction lysed showing marked fibrinolysis even before the corresponding recalcified plasma had started to clot. Table II tabulates the euglobulin lysis times of a number of

TABLE II
EUGLOBULIN AND PLASMA LYSIS TIME IN MINUTES BEFORE AND 105 MINUTES AFTER INJECTION OF VARIOUS AMOUNTS OF PYROGEN

No.	Euglobulin lysis time		Plasma lysis time	
	Before (min.)	After (min.)	Before (hr.)	After
1	130	45	>24	>24 hr
2	340	50	>24	>24 hr
3	167	51	>24	>24 hr
4	215	[illegible]	>24	>24 hr
5	[illegible]	52	>24	550 min
6	233	4	>24	542 min.
7	[illegible]	58	>24	114 min.
8	[illegible]	50	>24	45 min
9	220	15	>24	25 min
10	115	10	>24	50 min

patients receiving various doses of fibrinolysis inducing pyrogens. Again the euglobulin lysis time indicates the increased lysis tendency when the plasma clot shows nothing demonstrated by examples 1–4.

The cause for the development of marked fibrinolysis during diseases and some types of surgery is obscure. It may well be that certain tissues rich in plasminogen activator (1) release this substance into the circulation. Induction of fibrinolysis in man is a nonspecific reaction brought about by a variety of drugs. Table III lists some of these drugs and it can be seen that a number of nonenzymatic types produce fibrinolysis probably by triggering the release of the activator. The possibility of inducing fibrinolysis in humans by nonenzymatic drugs gives reason to hope that better and more effective compounds, free of side reactions, can be found which could produce lysis in man. In this respect testing of drugs for their action on the human fibrinolytic system should be-

ulins after their redissolution in buffered saline The reaction speed indicates the degree of fibrinolysis tendency In our experiments lysis times of less than 2 hours were considered to reflect increased lysis tendency The euglobulin method has four distinct advantages first it is applicable in uncoagulable blood i e due to heparin Second it permits a rapid differentiation of whether the uncoagulability is due to the

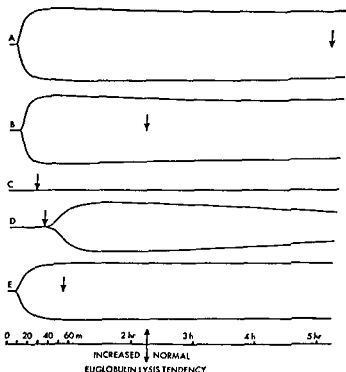


FIG 4 Serial coagulograms from citrated recalcified plasma and euglobulin lysis time determinations (arrows pointing downward) during open heart surgery A before anaesthesia B before by pass procedure C heparinization 7 minutes on pump D 5 minutes after 1 mg protamine per mg heparin E 30 minutes later after additional 0 5 mg protamine per mg heparin From von Kaulla and Schultz (17)

absence of fibrinogen or the presence of anticoagulants Third it permits rapid detection of increased fibrinolysis tendency thus providing clinically important information at an opportune time Fourth it is very sensitive reflecting increased lysis tendency in situations in which observations of a clot show nothing and other time consuming methods would have to be used for detection (17) Figure 4 demonstrates this The data showing serial thrombelastograms with their coordinated

come a routine procedure in all institutions concerned with the development of new therapeutic agents It is well known that nonenzymatic drugs inducing lysis in man are completely ineffective in animals and it was by this test study in man not animals that Eichenberger (4) discovered the powerful fibrinolysis inducing activity of protein free pyrogenic lipopolysaccharides We are able to confirm his results and can add more detailed observations (15) Figure 5 reproduces coagulo

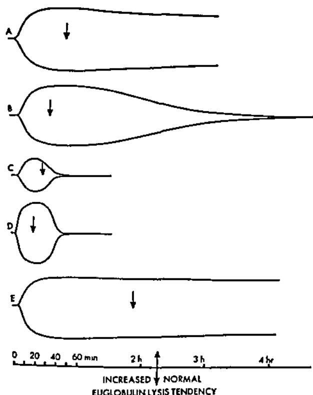


FIG 5 R C post paralytic polio Intravenous injection of 300 μg pyrogen Sa 1083 Serial coagulograms with coordinate euglobulin lysis time (arrows pointing downward) A obtained prior to pyrogen injection B C D and E 60 105 150 and 330 minutes after injection respectively From von Kaulla and Schultz (17)

grams together with the coordinated euglobulin lysis times (indicated by arrows pointing downward) of a patient who received 300 μg lipopolysaccharides intravenously The time relation of fibrinolysis to injection can be seen Coagulogram A was obtained before injection B C D and E 60 105 150 and 330 minutes respectively after injection Maximum fibrinolysis appears approximately 105–120 minutes after in

TABLE III

SUMMARY OF THE MOST IMPORTANT DRUGS PRODUCING FIBRINOLYSIS IN MAN

Drug	Intensity of action	Duration of action	Active in test tube	Mechanism of action	Remarks
Trypsin (i v)	Weak	Short	Yes	Direct digestion of fibrin. Activation of plasminogen.	Effect is uncertain
Plasmin (i v)	Strong	During infusion	Yes	Direct digestion of fibrin	
Streptokinase (i v)	Strong	During infusion	Yes	Activation of proactivator	
Acetylcholine (i v)	Strong	Few minutes	No	?	Effective near shock level
Epinephrine (s c)	Weak	Fraction of hours	No	?	
Irgapyrine (i v) Butazolidine (i v)	Very weak	Minutes to hours	No	?	Effect is uncertain
Paraaminobenzoic acid (oral)	Very weak	Few hours	No	?	Effect is uncertain
Novocain (i v)	Weak	Minutes to hours	No	?	Effect is uncertain
Protamine sulfate (i.v)	Weak to strong	Minutes	To some extent	Neutralizes anti plasmin? Activates plasminogen?	Effect is uncertain
Typhoid vaccine (not shock free)	Strong	Hours	No	?	Considerable side reactions

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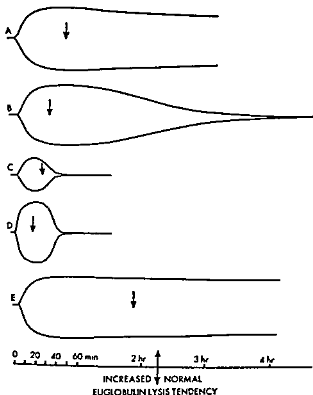


FIG 5 R.C post paralytic polio Intravenous injection of 300 μg pyrogen Sa 1083 Serial coagulograms with coordinate euglobulin lysis time (arrows pointing downward) *A obtained prior to pyrogen injection B C D and E 60 105 150* and 330 minutes after injection respectively From von Kaulla and Schultz (17)

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travenous injection The patient (in Fig 5) with liver cirrhosis and low prothrombin activity demonstrated a noticeably increased lysis tendency before the injection as indicated both by coagulogram and by euglobulin lysis time She responded with a very marked fibrinolytic reaction of long duration There was no bleeding Incidentally very marked fibrinolysis in human blood is not synonymous with bleeding

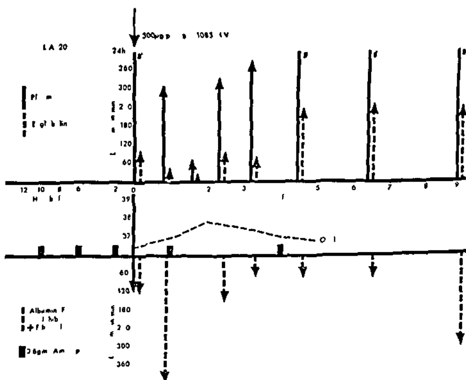


FIG 6 Intravenous injection of 300 µg of pyrogen Sx 1083 Trend of plasma lysis time (black arrows) euglobulin lysis time (dotted arrows pointing upward) and antifibrinolysin titer (dotted arrows pointing downward) Note rebound effect of euglobulin lysis time Note time scale From von Kaulla (15)

provided that no anticoagulant is present and no exogenous enzymes are involved After fibrinolysis had subsided in this patient the initial increase in lysis tendency as measured by the euglobulins had disappeared The temporary reduction of the spontaneous lysis tendency was consistently observed after fibrinolysis had been induced in humans by activating the fibrinolytic system with nonenzymatic stimuli This is also demonstrated by Fig 6 which shows combined measurement of plasma lysis time euglobulin lysis time and antifibrinolysin in another

patient who received intravenous pyrogens The euglobulins symbolized by dotted arrows pointing upward clearly reflect the trend of fibrinolysis tendency and it can be noted that their spontaneous lysis is definitely longer after the fibrinolytic reaction subsided, than before injection As already mentioned this indicates that the human blood has for a short time less tendency to lyse after it goes through a period of marked fibrinolysis From Fig 6 it can also be seen clearly that antifibrinolysin (symbolized by dotted arrows pointing downward) became reduced but did not completely disappear during maximum fibrinolysis It was found that the height of the preexisting antifibrinolysin titer had no bearing on the intensity or duration of pyrogen induced fibrinolysis Figure 6 shows also that the antifibrinolysin titer has no relation to the postfibrinolytic reduction of spontaneous lysis tendency A similar post fibrinolytic reduction of spontaneous lysis tendency was also observed after spontaneous lysis during surgery This will be shown later The causes of this temporary and incomplete refractoriness or refractory phase are unknown It persists for few hours following which the euglobulin lysis time returns to normal It can be broken through however by stronger stimuli as we observed during the pyrogen studies and as we suspect happens in surgical patients It might be assumed that the endogenous activator of plasminogen is involved in this phenomenon At present, it cannot be measured in the plasma however a method was developed to determine the quantity of plasminogen activator in the urine with the idea of obtaining more insight into its significance (16) In order to accomplish this it was essential to separate into two independent and unrelated steps the activation of plasminogen to plasmin from the action of plasmin itself on the substrate Table IV shows the steps of the procedure The fact that human urine activates the plasminogen of human plasma during rotation dialysis in the cold and that the resultant plasmin stays stable during this dialysis for hours was used to good advantage The yield of the accumulating plasmin was not reduced by concomitant plasmin inactivation It can be seen from this Table that the activation mixtures always contain 1 ml standard plasma but increasing amounts of urine are added in order to obtain various plasma urine proportions ranging from 1 1 to 1 10 With the plasma urine proportions of 1 3 and 1 4 the most plasminogen that can be activated under the conditions of the experiment is activated The activation was complete with the proportion 1 5 and no further activation could be obtained by increasing the plasma urine proportion to 1 10 (or by adding a potent urokinase preparation) Figure 7 gives a characteristic urokinase determination graph of the morning urine of a normal individual Figure 8 reproduces the average values of 12 nor

TABLE IV
STEPS IN UROKINASE DETERMINATION

Reagents	Procedure						Reaction
Fresh unfiltered urine +	1	2	3	4	5	10 ml	
Citrated plasma +	1	1	1	1	1	1 ml	
H_2O	19	18	17	16	15	10 ml	
	Each mixture dialyzed for 90 min in 8/32 cellophane tubing against running ice cold tap water						plasminogen —urokinase→ plasmin
	Euglobulins precipitated by CO_2 saturation separated by centrifugation and dissolved in						plasmin with euglobulin ↓ antiplasmin→
Phosphate buffer 1/15 *M* pH 7 4	1	1	1	1	1	1 ml	
	Add to mixture						
Casein 1% purified in phosphate buffer 1/15 *M* pH 7 4	4	4	4	4	4	4 ml	
	Incubate for 90 min 37 C						Digestion of casein by plasmin
Folin Ciocalteu	Determination of released tyrosine Representative values						
	13 7	21 7	26 5	27 7	28 5	30 0 μg	

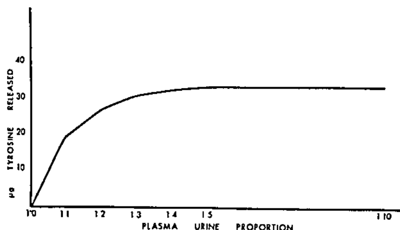


FIG 7 Typical urokinase determination graph of the morning urine of a normal individual

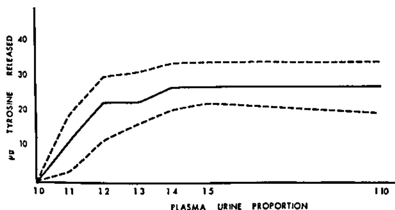


FIG 8 Average values for urokinase determination in the morning urine of 12 normal individuals The range for highest and lowest values is indicated

mal individuals together with the ranges encountered Figure 9 shows the urokinase excretion of patients with malignancies compared with that of a patient 7 days after removal of a kidney cyst We frequently observed a low excretion of urokinase independent from the urine concentration in malignancies and in kidney disorders even when there was a considerable protein excretion The significance of the lowered urokinase excretion is not clear yet but further observations may reveal more about the biological significance of the human fibrinolytic enzyme system On the other hand the urokinase excretion patterns observed

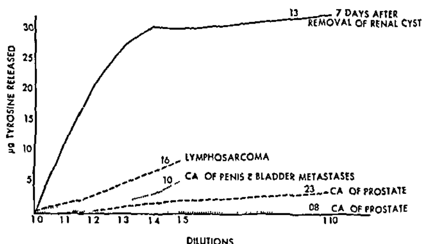


FIG 9 Urokinase excretion in 4 patients with malignancies and in 1 patient on the seventh day after the removal of kidney cyst Ciphers above curves indicate specific gravity of the urine

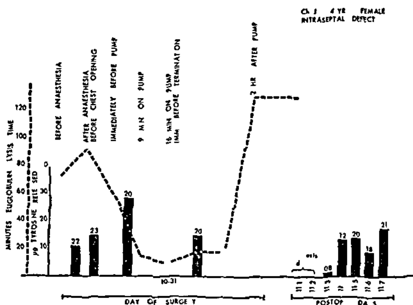


FIG 10 Trend of euglobulin lysis time (dotted line) and urokinase excretion (histograms) before during and after open heart surgery with pump oxygenator Ciphers on top of histograms indicate specific gravity of urine Drop in euglobulin lysis time reflects marked increase in fibrinolysis tendency Prolongation of post fibrinolytic euglobulin lysis time over prefibrinolytic control value indicates appearance of refractory phase There is an increase in urokinase excretion in relation to lysis

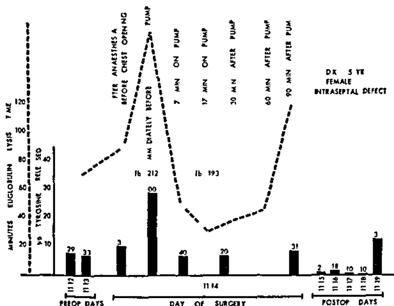


Fig 11 Trend of euglobulin lysis time (dotted line) and urokinase excretion (histogram) before during and after open heart surgery with pump oxygenator Cipher on top of histograms indicates specific gravity of urine Increase in euglobulin lysis time reflects transfusion with blood having a very minute spontaneous lysis tendency Drop indicates marked increase in lysis tendency There is an increase of urokinase excretion in relation to lysis

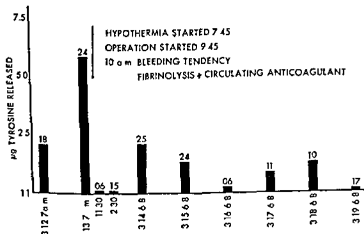


Fig 12 Trend of urokinase excretion before during and after operation for porto-caval shunt complicated by fibrinolysis plus circulating anticoagulant Ciphers on top of histogram indicate specific gravity of urine Note time sequence

in man in connection with marked increased fibrinolysis tendency are easier to understand Characteristic findings are shown in Fig. 10–13 which reproduce serial urokinase excretion studies The plasma urine proportion 1 1 which we consider as the most accurate indicator for urokinase activity was used Figure 10 shows the urokinase excretion in a child undergoing open heart surgery for repair of ventricular septal defect with the use of a pump oxygenator The sudden rise in urokinase excretion related to the appearance of marked increase in fibrinolysis

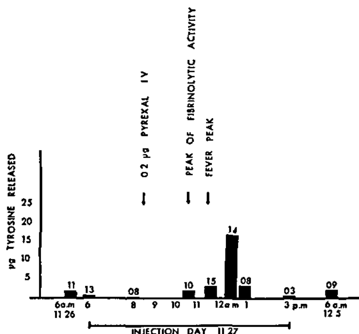


Fig 13 Trend of urokinase excretion before during and after pyrogen induced fibrinolysis in man Ciphers on top of histograms indicate specific gravity of urine Note time scale

tendency reflected by the drop in euglobulin lysis time (dotted line) is clearly seen as is a postoperative drop in urokinase excretion Here again the euglobulin lysis time after the fibrinolytic phase is longer than that of prefibrinolytic control samples Figure 11 shows a similar fibrinolytic patient Again the rise of urokinase excretion closely re lated to fibrinolysis and the postoperative drop of this excretion is prominent Figure 12 is obtained from a patient operated upon for porto caval shunt As soon as surgery was begun he hemorrhaged due to transient fibrinolysis combined with a circulating anticoagulant The

sudden rise of urokinase excretion before the appearance of lysis and subsequent drop was postoperatively observed Figure 13 shows serial urokinase excretion studies in a patient with a Hufnagel valve in whom fibrinolysis was induced by intravenous pyrogens Here the peak of urokinase excretion appeared after fibrinolysis had subsided It is as

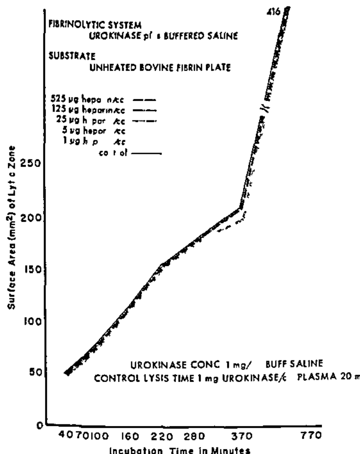


FIG 14 Effect of human urokinase on unheated bovine fibrin plates in presence of various amounts of heparin Surface areas of fibrinolytic digestion zones are plotted against incubation time From von Kaulla and McDonald (15a)

sumed that the action of the pyrogens on the kidney i e vasoconstriction followed by vasodilatation might have caused this particular excretory pattern The last four figures shown demonstrate that increased urokinase excretion is closely related to the appearance of marked

fibrinolysis tendency in man perhaps indirectly reflecting the release of plasminogen activator from the tissues into the circulation We presently feel that the true indicator for urokinase release is not so much the absolute amount of urokinase excretion at any one time but a clear cut increase in respect to precedent values It is not known whether the increased urokinase excretion reflects a lowering of the kidney threshold for urokinase or a spilling over of increased kinase of the blood

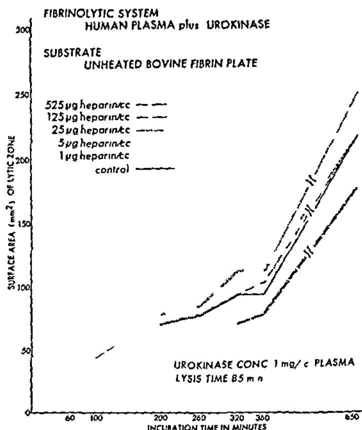


Fig 15 Effect of human urokinase on unheated bovine fibrin plates in presence of undiluted human plasma together with various amounts of heparin Surface areas of fibrinolytic digestion zones are plotted against incubation times From von Kaulla and McDonald (15a)

It might be worthwhile to try to enhance the fibrinolytic potentiality of the blood therapeutically by reducing or inhibiting the excretion of urokinase by the normal kidney This effect could be combined with the use of nonenzymatic agents which increase the fibrinolytic activity

by triggering the release of the activator from the tissues Another path way might be to increase the accessibility of fibrin for the fibrinolytic enzyme This mechanism has not been taken earnestly into considera tion

Our experiments suggest that heparin might do this In these studies we employed urokinase using the fibrin plate method of the Astrup group (2, 7) The plate method allows evaluation of the fibrinolytic activity on preformed fibrin by measuring the size of fibrinolytic diges tion zones It was found that small amounts of heparin (1–5 μg /ml)

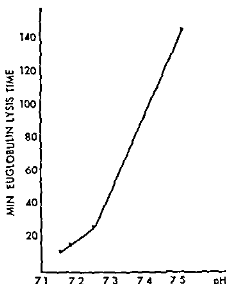


FIG 16 Relationship between maximum euglobulin lysis time and the pH of the blood in the immediate post pump period Average values of groups of 3 pa tents each with similar lysis times are plotted against their average blood pH

enhance fibrinolysis whereas large ones (above 100 μg /ml) inhibit it A cofactor contained in the albumin fraction is required for this effect These observations appear to settle the old controversy concerning heparin influence on human fibrinolysis This is shown by the next two Figures On Fig 14 growth of these digestion zones in mm² brought about on fibrin plates by urokinase in presence of various heparin con centrations is plotted against incubation time There is no difference between the growth rate of the control zones and those containing various heparin concentrations Figure 15 shows what happens if un diluted human plasma is added to the same system The growth of the zones containing small amounts of heparin starts earlier and proceeds

faster than the control and the growth of the zones with the large amounts of heparin is inhibited This indicates that fibrinolysis is enhanced by the small amounts of heparin and clearly is inhibited by the large ones

In closing it should be mentioned that we have suggestive evidence for a relationship between the pH of the blood and fibrinolysis as shown in Fig 16 Here the average values for groups of 3 surgical fibrinolytic patients each with comparable euglobulin lysis time at the peak of the fibrinolytic reaction are plotted against the average pH of their blood at the same time It was observed in our surgical patients that fibrinolysis did not develop when the perfusion rate was high thus making it easier to keep the pH on the more alkaline side In general it was seen from a study based on twelve patients perfused with pump oxygenator that a low pH of the blood may or may not favor fibrinolysis but once fibrinolysis is induced a low pH of the blood seems to enhance and prolong this phenomenon

At present we are only at the very beginning of the understanding of the significance of the human fibrinolytic system but considerable progress has been made It is conceivable that an effective controlled activation of this system for therapeutic or prophylactic purposes can be achieved in the near future without resorting to exogenous enzymatic material

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Discussion

Dr Vitale Dr Sherry in your last slide you had plasminogen plasmin and an activator then in capital letters "fibrinogen protected This is protection by what?

Dr. Sherry Fibrinogen is protected from the action of plasmin by large amounts of antiplasmin in the circulation thus rapidly inactivating any plasmin which is formed The amount of inhibitor or antiplasmin present is exceedingly large for the amounts of plasmin which may be formed under these circumstances

Dr. Wexler Angioplasm is probably formed at the embryonic level and we think of this mesenchymal tissue as a mass of primitive reticular cells which go through a phase of rounding up Those on the perimeter form endothelial cells Those in the center become the various formed elements Things are static at first until the tissue forms interstitial fluid resulting in a flow of blood

What is peculiar about the surface of this young endothelial cell which prevents clotting? The mechanism can go wrong as in the vitamin E deficient mothers who give birth to young with a defect at this point in embryological development Clotting occurs in these angioblastic islets

I like to look at life through adrenal-colored glasses and Dr Sherry I noticed you mentioned epinephrine electroshock, pyrogens—all excellent stimulators of the pituitary adrenal system I felt that you thought these stimuli produce the activator by means of a neural mechanism I have been interested in the effects of pyrogens (Piromen) on the pituitary adrenal system particularly From the endocrine standpoint the changes in adrenal steroids the eosinopenia—the typical adrenal changes—can be correlated with appearance of fever and chill

Dr von Kaulla spoke about the dose of pyrogen having a relationship to the clinical changes If a patient is given 5 to 25 μg of Piromen a chill and fever result, with little evidence of adrenal cortical stimulation If dosage is increased to 100 μg fever did not appear i e an intense release of hydroxycorticoids must mask the fever At this level there seems to be a hypothalamic pituitary threshold with adrenal response consisting of increased hydrocorticoid production and eosinopenia

This material does as you say first produce constriction of the renal artery and then dilation followed by actual renal hypertrophy

In rabbits fed cholesterol we produced typical atheromatous lesions—the foam cell lesion—but those fed with cholesterol plus injection of Piromen did not develop lesions

In studies on experimental frostbite we were concerned with the development of gangrene and thrombosis A frozen rabbit s ear will hang dependent because of massive edema There is thrombosis and if you have dipped the ear into the freezing solution, there is a remarkably sharp line of demarcation between the immersed portion and the remaining portion.

Animals receiving rapid thaw therapy alone develop this edema The ear hangs dependent becomes dried and mummified, and separates by natural amputation

Baxter Laboratories Inc Morton Grove Illinois

In animals similarly treated but given Piromen the edema clears rapidly and finally the ear stands up in the erect position just like its mate It is re epithelialized in that it is unusually heavily covered with hair demarcating the frozen portion from the unfrozen indicating to me a rich vascular supply and certainly no sign of thromboses or occlusion

In rabbits that received only rapid thaw therapy 5 of 30 whose ears survived after freezing the unfrozen portion was well maintained and the frozen portion was leathery and tough If Piromen was given to those whose ears managed to hang on resorption of the cartilage back to the original aural plate with a complete resorption of cartilaginous material occurred with formation of new vascular channels

Dr Ratnoff It is quite obvious that under normal conditions the endothelial surface does not offer a stimulus for the initiation of clotting I would assume that the newly formed angioblastic islets don t either

Dr von Kaulla Fibrinolysis can be inducted in man by injecting such substances as pituitary extract It is an unspecific phenomenon

In relation to fibrinolysis and fever therapy in 80 patients we studied the peak of fibrinolytic activity occurred before the fever peak The two peaks were dissociated Fibrinolysis occurred roughly with the chilling phase and at the time of eosinopenia If the fever was suppressed by antipyretics successfully (this is not always possible) the fibrinolytic reaction remained

Dr Wexler Dr Jailer has shown that if antipyretics are used with pyrogens to cancel the pyretic effect an endocrine effect can still be shown

Dr Sherry Initially our experiments were designed to study the biochemical mechanism by which thrombus dissolution occurred rather than an investigation of physiological mechanisms We concluded that one had to consider the lysis of a clot as representing a coupled reaction The clot contains plasminogen as well as fibrin The first step is activation of the clot plasminogen followed by fibrin dissolution However these studies were provocative and I mentioned them for speculation I do not believe the electroshock experiments necessarily prove a neural mechanism alone Perhaps a neurohumoral mechanism might also be concerned We were impressed that the release of fibrinolytic activity happened within a minute following electroshock secondly that injection of adrenal steroids per se does not increase fibrinolytic activity and if anything appears to increase the antiplasmin content of blood thirdly that fibrinolytic activity appears in the veins of an ischemic arm immediately following electroshock

Also studies by Dr Kwaan *et al* on volunteers in whom cuffs were applied to the arm preceding injection of acetylcholine either intravenously into a distended vein or paravenously resulted in increased fibrinolytic activity within that local segment of vein They also showed that serotonin had a similar effect—atropine would block the effects of acetylcholine or serotonin

Dr Ratnoff In Dr Beggs studies in 1947 (R Beggs R G Macfarlane and J Pilling *Lancet* 1 402 1947) she clearly demonstrated the lysis accelerating effects of adrenaline and exercise in patients with Addison s disease

Dr Ambrus Dr Ratnoff how do you visualize the absorption of Hageman inhibitor by various tissues and the physiological initiation of the clotting mechanism? What would be the relative importance of possible absorption of the Hageman factor by injured tissue and what would be the relative importance of disintegration of platelets which we teach our medical students starts clotting?

The phenomenon that puzzled Dr von Kaulla of a possible second clotting of

apparently lysed clots has bothered us also We find in preliminary work that if plasmin hydrolyzes fibrin it can definitely not clot again However one may dissolve the majority of the members of a fibrin meshwork and leave some fibers free to appear in the supernatant fluid these are the members which then under various physicochemical conditions can again aggregate

I would also like to support Dr von Kaulla s observation with heparin *in vitro* We have undertaken similar experiments *in vivo* in experimental animals and patients Heparin greatly potentiates the fibrinolytic effect of plasmin therapy If we treat a clot by daily administration of plasmin we may dissolve part of the clot but plasmin very rapidly disappears from the circulation and the remaining clot is then free for extension by apposition When we administer the next treatment we not only have to dissolve the remainder of the clot but also the clot which formed by apposition The latter can be reduced by heparinization In some patients treated with fibrinolytic therapy the clot gradually disappeared but at the same time new clots appeared elsewhere In other words while plasmin is able to dissolve clots it will not prevent new clot formation I think that therapeutically we will have to combine fibrinolytic therapy with anticoagulants

Dr Sherry said that part of the specificity of fibrinolytic enzymes may be due to the fact that they activate plasminogen which is caught in the meshwork of the clot However we should not neglect a second mechanism involved in the specificity This is the selective adsorption of plasmin on the surface of fibrin while the antiplasmin is not adsorbed In the circulating blood plasmin is inhibited by antiplasmin but on the fibrin plasmin is selectively adsorbed and antiplasmin is not therefore a high active concentration can be formed

From studies with radioactive enzymes we find there may be a competition between fibrin and the antiplasmin plasmin complex for plasmin so that antiplasmin may really act to transport plasmin in an inactive form to existing clots

If one injects labeled plasmin into a dog fibrinolytic activity very rapidly disappears from the blood yet radioactivity due to the labeled plasmin remains at considerable levels in the blood and apparently plasmin is now circulating in an inactive plasmin antiplasmin complex

On the surface of the clot there seems to be a concentration of plasmin The clot will show signs of lysis between 4 and 24 hours after injection of plasmin although all plasmin activity disappeared from the blood within 1 hour The clot will lyse whether we leave it in the dog or whether we take it out and incubate it in a test tube

Dr Sherry s own experiments demonstrate this active adsorption of plasmin on fibrin and I feel that this is a second mechanism in the selective action of plasmin upon fibrin While we may debate which of the two mechanisms is the more important I think both are involved

Finally we have treated patients with coexistent thromboembolic disorders and acute hemorrhagic complications (such as gastrointestinal bleeding due to carcinoma or large post radiation wounds) In these cases we were able to dissolve clots without producing significant hemorrhage

Dr Sherry I would agree that thrombi may be lysed either by activation of intrinsic plasminogen or by the surface action of plasmin Our data suggests that activation of clot plasminogen is a more sensitive mechanism for thrombus dissolution than lysis by circulating plasmin

Unfortunately some of the preparations referred to as plasmin are not really plasmin alone For example the preparations referred to by Dr Ambrus represent

In animals similarly treated but given Piromen the edema clears rapidly and finally the ear stands up in the erect position just like its mate It is re-epithelialized in that it is unusually heavily covered with hair demarcating the frozen portion from the unfrozen indicating to me a rich vascular supply and certainly no sign of thromboses or occlusion

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DR VON KAULLA Fibrinolysis can be inducted in man by injecting such substances as pituitary extract It is an unspecific phenomenon

In relation to fibrinolysis and fever therapy in 80 patients we studied the peak of fibrinolytic activity occurred before the fever peak The two peaks were dissociated Fibrinolysis occurred roughly with the chilling phase and at the time of eosinopenia If the fever was suppressed by antipyretics successfully (this is not always possible) the fibrinolytic reaction remained

DR WEXLER Dr Jailer has shown that if antipyretics are used with pyrogens to cancel the pyretic effect an endocrine effect can still be shown

DR SHERRY Initially our experiments were designed to study the biochemical mechanism by which thrombus dissolution occurred rather than an investigation of physiological mechanisms We concluded that one had to consider the lysis of a clot as representing a coupled reaction The clot contains plasminogen as well as fibrin The first step is activation of the clot plasminogen followed by fibrin dissolution However these studies were provocative and I mentioned them for speculation I do not believe the electroshock experiments necessarily prove a neural mechanism alone Perhaps a neurohumoral mechanism might also be concerned We were impressed that the release of fibrinolytic activity happened within a minute following electroshock secondly that injection of adrenal steroids per se does not increase fibrinolytic activity and if anything appears to increase the antiplasmin content of blood thirdly that fibrinolytic activity appears in the veins of an ischemic arm immediately following electroshock

Also studies by Dr Kwaan *et al* on volunteers in whom cuffs were applied to the arm preceding injection of acetylcholine either intravenously into a distended vein or paravenously resulted in increased fibrinolytic activity within that local segment of vein They also showed that serotonin had a similar effect—atropine would block the effects of acetylcholine or serotonin

DR RATNOFF In Dr Beggs studies in 1947 (R Beggs R G Macfarlane and J Pilling *Lancet* 1 402 1947) she clearly demonstrated the lysis accelerating effects of adrenaline and exercise in patients with Addison s disease

DR AMBRUS Dr Ratnoff how do you visualize the absorption of Hageman inhibitor by various tissues and the physiological initiation of the clotting mechanism? What would be the relative importance of possible absorption of the Hageman factor by injured tissue and what would be the relative importance of disintegration of platelets which we teach our medical students starts clotting?

The phenomenon that puzzled Dr von Kaulla of a possible second clotting of

effect on the fibrinolytic system In retrospect the impressive thing in our management of malignant hypertensives a number of years ago was not the hypotensive effect that pyrogen produced but the effect on the blood vessel lesions The eyegrounds cleared up and the patient reverted from the malignant phase to ordinary essential hypertension At that time this seemed so unlikely that most people did not believe it Whether this material actually dissolved something in the blood vessels or simply kept it from being deposited progressively over a period of time I have no idea But it did show I believe for the second time that malignant hypertension was reversible The first was when we noticed that the eyegrounds cleared up after Adson and Allen's lumbodorsal sympathectomy

CHAIRMAN ALLEN Long ago we indicated that when one induced fever in the treatment of thromboangiitis obliterans the effects—from the standpoint of restoration of circulation and relief of pain—lasted far beyond the period of pyrexia ranging from 2 to 4 days It never occurred to us that might be owing to fibrinolysis I assume it might be

We have been much interested in the fact that if one puts blood into a glass tube or in a nonwettable silicone tube or paraffine tube one gets very widely diverse results in coagulation time How about using a blood vessel as the tube Dr Ratnoff?

DR RATNOFF Dr J Lister did this experiment about 100 years ago (*Proc Roy Soc* **12** 580 1862–1863) He took the vessel out tied off the two ends put it under saline for a day or so then opened it up The blood within was still fluid We have not progressed much on this problem to date I do not believe there has been any work done in the light of this modern viewpoint concerning the role the vessel walls play per se We do all our thinking by extension rather than by experiment which perhaps saves us from innumerable corrections of our hypotheses that experimentation would provide Parenthetically John Hunter first studied fibrinolysis around 1790

human plasminogen preparations activated with either streptokinase or urokinase This mixture of activator and plasmin is injected into animals and is referred to as plasmin It is not plasmin per se but is a mixture of plasmin plus activator and in addition has an enhanced capacity for activating animal plasminogen Under these circumstances it is difficult to define quantitatively what each component is contributing to the lysis of an intravascular clot

DR VON KAULLA I can support Dr Ambrus observations Fibrinolysis can be induced in patients who might have a bleeding tendency In our 80 pyrogen treated patients of 40 *women studied during the first to fourth postpartum day* who had bloody lochiae their blood content was not increased by fibrinolysis As Dr Ambrus said the clot lysis continues when the fibrinolytic activity has disappeared If we treat superficial thrombophlebitis postpartum thrombophlebitis and combine in the therapeutic procedure one single anticoagulant (heparin or Tromexan) with one single pyrogenic injection for producing strong fibrinolysis then we can follow clot disappearance The regression of the clot goes on in the hours after fibrinolytic activity has disappeared

We have used different methods but agree with Dr Sherry that the activator is the thing on which to concentrate

DR SHERRY One should recognize when discussing treatment of thrombo embolic disease that approaches may have to vary depending on the particular problem We attempt to maintain very high levels of thrombolytic activity i e approximately 100 µg of fibrin lysed per milliliter of plasma per hour Thus if we are dealing with an acute arterial occlusion it would be very important to dissolve the thrombus as quickly as possible or before irreversible changes set in In therapy for venous thrombi the needs may not be as acute and the induction of a much less active fibrinolytic state may be effective over a longer time

Finally we can conceive of prophylactic fibrinolytic therapy which would require only small increase in fibrinolytic activity I agree with Dr von Kaulla that the ultimate in fibrinolytic therapy will be a pharmacological agent which can control the level of circulating activator in the blood

DR DRILL Did Dr von Kaulla mention that nonenzymatic materials would have to be studied in man rather than animals? Is there a system available to study fibrinolysis in animals with some chance of transferring the results or drawing conclusions to man?

DR VON KAULLA I know of no animal and of no fibrinolytic system which can be used except the human body to study compounds which trigger the release of activators I know of no compounds which induce fibrinolysis both in animals and in human beings except injection of active enzymatic material Acetylcholine and other materials like pyrogens do not work in animals We have experience with dogs and rabbits and I think Dr Ambrus has studied monkeys

DR RATNOFF Certainly in dogs postoperatively you see fibrinolysis Canine systems are richer in inhibitory material and therefore one is operating at a different level

DR BERLINER Regarding Dr Wexler's question Dr White reminded us that ACTH has an extra adrenal effect Did you test ACTH Dr Sherry?

DR SHERRY No we tested hydrocortisone but not ACTH

DR VITALE Dr Sherry why could not plasmin act by digesting thrombin and protecting fibrinogen instead of calling upon an antiplasmin?

DR SHERRY We have evidence that it does not digest thrombin

DR PACE Dr von Kaulla mentioned the fact that pyrogen had a profound

effect on the fibrinolytic system In retrospect the impressive thing in our management of malignant hypertensives a number of years ago was not the hypotensive effect that pyrogen produced but the effect on the blood vessel lesions The eyegrounds cleared up and the patient reverted from the malignant phase to ordinary essential hypertension At that time this seemed so unlikely that most people did not believe it Whether this material actually dissolved something in the blood vessels or simply kept it from being deposited progressively over a period of time I have no idea But it did show I believe for the second time that malignant hypertension was reversible The first was when we noticed that the eyegrounds cleared up after Adson and Allen s lumbodorsal sympathectomy

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Immunochemistry and Its Relationship to Atherosclerosis

DAVID GITLIN

Children's Hospital, Harvard Medical School, Boston, Massachusetts

Any relationship between this particular discussion and the title is purely coincidental. I would, however, like to discuss some of the points that have been brought up earlier in this symposium and in so doing obtusely indicate the potential of immunochemistry that may be applied to the study of atherosclerosis.

It is essential to emphasize first that the plasma proteins, fibrinogen included, are not restricted to the circulation (1, 4, 7, 10). This is now

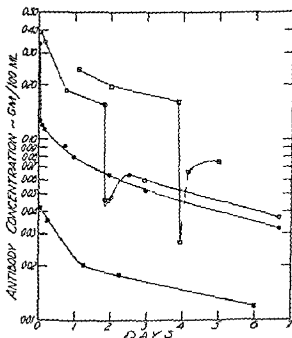


FIG. 1. Plasma concentration of rabbit antipneumococcus type 3 passively transferred intravenously to groups of nonimmune rabbits. (Reprinted with permission from *Science* **118**, 301, 1953.) □ exchange transfusion with normal rabbit blood at end of fourth day; ○ pneumococcus polysaccharide type 3 given intravenously at end of second day; ● pneumococcus polysaccharide type 3 given intravenously after 20 minutes; ■ antibody-containing plasma obtained from exchange transfusion of first group injected into other rabbits.

well known and is perhaps most easily demonstrated by the experiment represented by Fig 1 A group of rabbits were injected intravenously with rabbit antiserum prepared against pneumococcus polysaccharide type 3 it should be noted that the antiserum was prepared in one group of animals and then given passively to the experimental animals In this case a specific gamma globulin antipolysaccharide type 3 could be detected by virtue of its capacity to react with its homologous antigen polysaccharide type 3 and exogenous labels were not used The loga

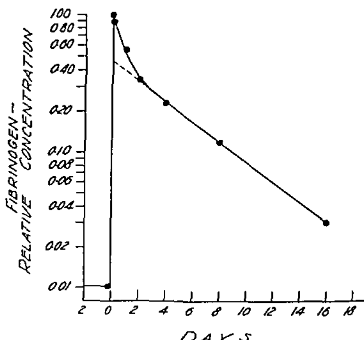


FIG 2 Plasma disappearance of fibrinogen after intravenous infusion in a child with congenital afibrinogenemia (Reprinted with permission from *Blood* 8 679 1953)

rithm of the concentration of antibody in the plasma of the recipient animals was plotted against time and one observes the familiar plasma disappearance curve for a labeled homologous plasma protein Initially the concentration of antibody falls rapidly and then more slowly finally reaching a phase of exponential decline The phase of relatively slow decline represents the removal of antibody from the plasma by catabolism or degradation

On the other hand the phase of rapid disappearance of antibody from the circulation is attributable to the diffusion of antibody from the vascular system into the interstitial fluids The antibody present in the

extravascular extracellular space is neither stored nor does it remain stagnant but is rapidly returned to the vascular system the amount returned in the steady state equals the amount diffusing from the circulation The amount of specific protein in the interstitial fluids during the steady state is about one and a half times that present in the vascular system If the antibody in the circulation is removed either by exchange transfusion with normal rabbit blood or by reaction with specific antibody and rapid removal of antigen antibody complexes by the reticuloendothelial system return of antibody into the plasma from the interstitial fluid is readily demonstrated

If human fibrinogen again unlabeled is given intravenously to children with congenital afibrinogenemia and followed immunochemically the behavior of fibrinogen is similar to that noted for specific rabbit antibody in rabbits (Fig 2) The fibrinogen diffuses into the extravascular fluids and in the steady state about half of the total fibrinogen in the body is present in the vascular system and about half is present in extravascular extracellular fluids This is true in normal man for all of the plasma proteins thus far studied

The diffusion of fibrinogen into the interstitial fluids can be shown by the fluorescent antibody method of Coons and Kaplan (2) In this method an antibody is prepared in animals against a specific antigen in this case fibrinogen The antifibrinogen antibody prepared in rabbits was labeled with fluorescein and used to study sections of human tissue cut at −18 to −30°C The tissue sections were flooded with labeled antibody and after a given time necessary to allow antibody to react with fibrinogen in the sections excess antibody was washed from the sections By examination of the sections under the ultraviolet microscope the distribution of fibrinogen was ascertained by the localization of the specific fluorescence of the labeled antibody

In Fig 3 A and D are shown sections from skin and muscle biopsies respectively "stained for fibrinogen using this method in a child with congenital afibrinogenemia no fibrinogen was apparent Other plasma proteins however such as albumin (Fig 3 B and E) could be demonstrated Twenty four hours after the intravenous infusion of human fibrinogen fibrinogen can be demonstrated in the connective tissue fluid in skin (Fig 3C) and muscle biopsies (Fig 3F)

In normal man fibrinogen and other plasma proteins are present in the connective tissues at all times and these are in dynamic equilibrium with the homologous plasma proteins in the blood vessels On the other hand fibrin does not appear to be a component of normal tissues at least if present it is not readily demonstrated by the fluorescent antibody method The fibrinogen observed in tissue sections is easily re

moved by washing the sections in saline prior to reacting them with labeled antibody

The fluorescent antibody method has been applied to a study of the so called fibrinoid degeneration of collagen In a study of fibrin clots

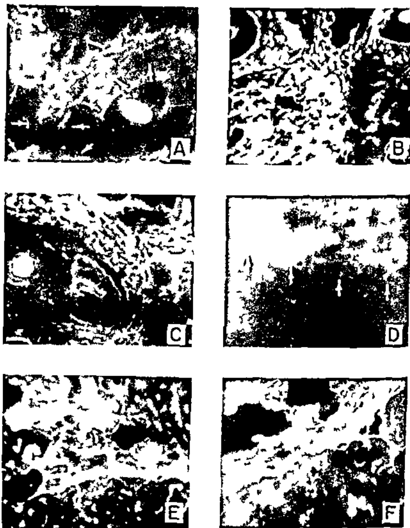


Fig 3 Sections of biopsies from a patient with congenital afibrinogenemia A to C are skin biopsies D to F are muscle biopsies White areas indicate fluorescence and hence sites of localization of protein studied All × 200 (Reprinted with permission from *Blood* 8 679 1953) A and D No fibrinogen in connective tissue (area outlined by arrows) Fluorescence of hair shaft and elastic fibers in A is nonspecific B and E Same biopsies as A and D respectively stained for albumin C and F Stained for fibrinogen 24 hours after fibrinogen infusion

formed *in vitro* from purified fibrinogen and thrombin it was soon learned that the usual histochemical dyes used to detect fibrin in tissues would react with fibrin *only* when *formed* from fibrinogen under certain conditions (6) Such dyes were unsuitable for the detection of fibrin in tissues they frequently gave a negative reaction for fibrin when fibrin could be demonstrated to be present by the fluorescent antibody method As we have heard before with collagen the stainability of fibrin with dyes is dependent upon the medium in which the fibrinogen is converted to fibrin The fluorescent antibody method involves only

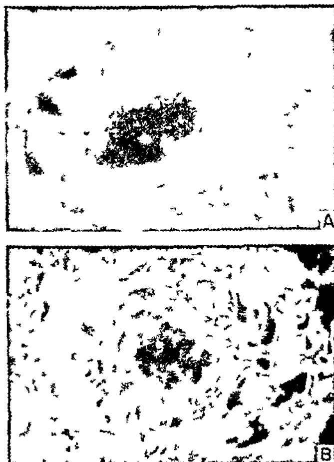


FIG 4 A F[illegible] proliferating intima of a small renal artery of a patient with polyarteritis nodosa × [illegible]10 B Fibrin in necrotic wall of another renal artery of same section as A × [illegible]10 (Reprinted with permission from *Am J Pathol* **33** 55 1957)

the specific reaction of fibrin with its specific labeled antibody and this is not readily influenced by the conditions under which the conversion or formation of fibrin takes place

In the tissue sections to be shown next fibrinogen has been washed

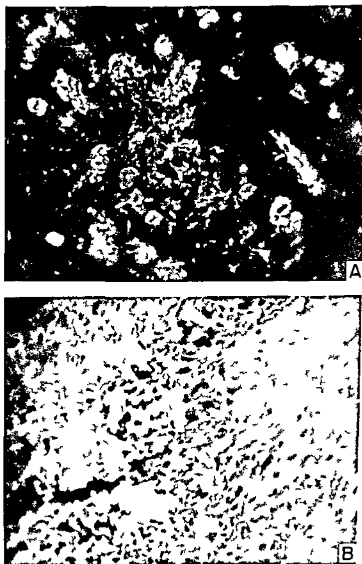


FIG 5 A Necrotic center of a whorl of reticular collagen in a rheumatoid nodule × 100 B Diffuse deposition of fibrin strands intermingling with collagen at periphery in a rheumatoid nodule × 210 (Reprinted with permission from *Am J Pathol* 33 55 1957)

from the sections prior to reacting with fluorescein labeled antifibrin antiserum since fibrinogen cross reacts with antifibrin antibodies The poor quality of definition is readily understood when one remembers that these sections were unfixed immersed in a variety of aqueous solutions and finally mounted in glycerol As before the white areas in the black and white photomicrographs indicate fluorescence (9)



FIG 6 Fibrin in thickened intima of a coronary artery of a patient with rheumatoid arthritis × 210 (Reprinted with permission from *Am J Pathol* 33 55 1957)

In Figs 4A and 4B are sections of renal arteries from a child who succumbed with polyarteritis fibrin was demonstrated readily in the intima of these vessels In Fig 4A the internal elastic membrane is fragmented the membrane being revealed by its characteristic intense blue fluorescence in contrast to the yellow green fluorescence of labeled antibody

In Figs 5A and 5B fibrin is demonstrated in the central areas of rheumatoid nodules These nodules were negative for fibrin using the usual dye techniques In Fig 5A the nodule has apparently undergone a series of reactions surrounding the fibrin center is loose collagen in

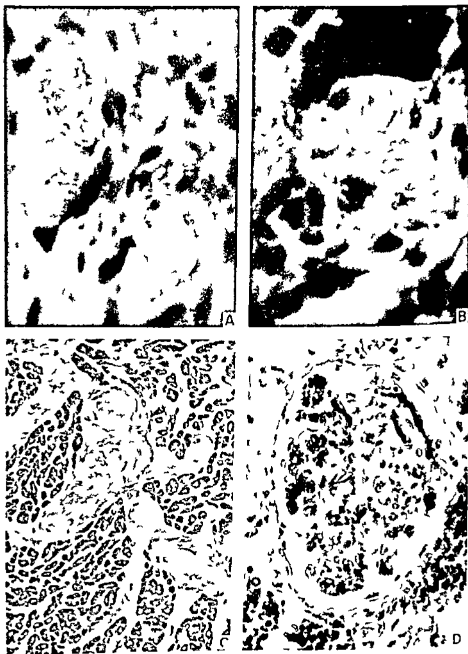


Fig 7 A Fibrin in thrombi in myocardium of a patient with thrombotic thrombocytopenic purpura × 210 B Fibrin in thrombi in renal glomeruli of a patient with thrombotic thrombocytopenic purpura × 210 C Similar section as A stained with phosphotungstic acid hematoxylin showing negative reaction for fibrin in the thrombi × 210 D Similar section as B stained with phosphotungstic acid hematoxylin showing a positive reaction for fibrin in the thrombi × 470 (Reprinted with permission from *Am J Pathol* 33 251 1957)

which dense collagenous bundles remain and fibrin can be detected in some of the dense bundles as well In many of these sections with close comparison of sections stained by the usual dyes and those stained with fluorescent antibody a *dye* positive fiber will suddenly stop in its positive reaction and yet the entire fiber or mass will be positive for fibrin with the fluorescent antibody method

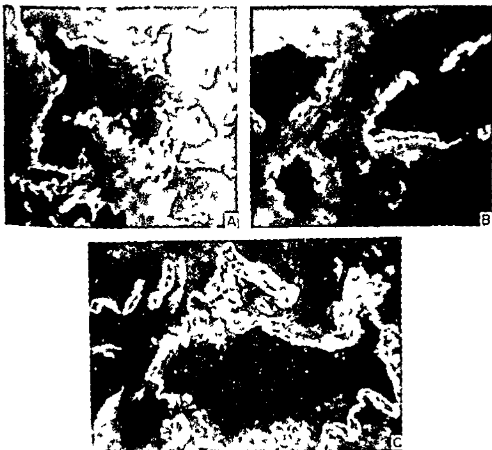


Fig 5 A and B Fibrin in pulmonary hyaline membranes of newborn children × 100 (Reprinted with permission from *Pediatrics* 1 64 19 6) C Fibrin in pulmonary hyaline membrane of patient with uremic pneumonitis × 210 (Reprinted with permission from *Am J Pathol* 33 55 1957)

A section of a coronary artery from a patient with rheumatoid arthritis is shown in Fig 6 Fibrin is present throughout the proliferating intima

Thrombotic thrombocytopenic purpura in children appears to be a

human analog of the generalized Schwartzman reaction Sections from children who have succumbed to this process revealed that the bulk of the thrombi found in the blood vessels as for example those in the myocardium (Fig 7A) or renal glomeruli (Fig 7B) is fibrin (3) Yet these thrombi were frequently negative for fibrin using histochemical dyes (Fig 7C) although some were positive (Fig 7D)

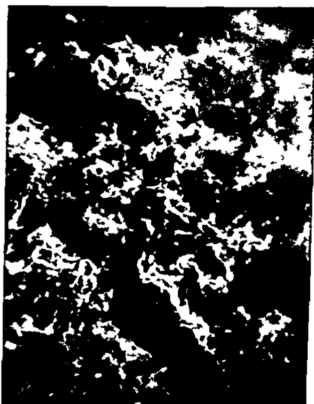


FIG 9 Rat fibrin in area of injection of carrageenin in rats × 210

Under certain conditions fibrin may be deposited in pulmonary alveoli and alveolar ducts In hyaline membrane disease of the newborn a large part of the pulmonary membrane appears to be fibrin (Fig 8 A and B) It is formed when fibrinogen diffuses out of the capillaries as part of an effusion and is then converted to fibrin the conversion occurring presumably through the action of a thromboplastic substance in amniotic fluid aspirated by the infant during delivery (5) Pulmonary hyaline membranes however may also occur in uremic pneumonitis (Fig 8C) in this case cellular necrosis would appear to release the thromboplastic substances for the conversion of fibrinogen to fibrin

A final remark before leaving the subject of fibrin. Fibrin deposition in areas of inflammation is to be expected. In the carrageenin experiments that were referred to earlier in this symposium the first thing that takes place is inflammation with the deposition of fibrin (Fig. 9); this is later replaced by collagenous tissue.

I would like to say something about the lipoproteins since we appear to have been using the term "cholesterol" extremely loosely. It should be remembered that the cholesterol of plasma is not free but is

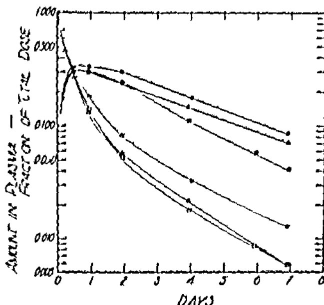


Fig. 10. Plasma disappearance of radioactivity from S_f 10 to 200 lipoproteins labeled with I^{131} and injected intravenously into three normal adults (black symbols) and the appearance of radioactivity in S_f 3 to 9 lipoproteins of these subjects. (Reprinted with permission from J. Clin. Invest. 37, 172, 1958.)

associated with the lipoproteins. The lipoproteins as we all know can be differentiated according to density and can be separated nicely in density gradient tubes in the ultracentrifuge. I wish to indicate only that there may be instances in which one protein may be converted to another and yet only one of the proteins may be of importance in the topic under discussion.

If I^{131} labeled S_f 10 to 200 lipoproteins are injected into humans (8) the label becomes increasingly associated with lipoproteins of S_f 3 to 9 (Fig. 10). The conversion of S_f 10 to 200 lipoproteins to those of S_f 3 to 9 appears to be a heterogeneous process. The metabolic behavior of the very high density lipoproteins, the α lipoproteins, is different from that

human analog of the generalized Schwartzman reaction Sections from children who have succumbed to this process revealed that the bulk of the thrombi found in the blood vessels as for example those in the myocardium (Fig 7A) or renal glomeruli (Fig 7B) is fibrin (3) Yet these thrombi were frequently negative for fibrin using histochemical dyes (Fig 7C) although some were positive (Fig 7D)

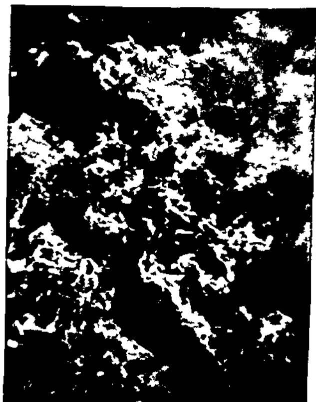


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Discussion

Dr Page This brings up the important and obvious problem of whether the immunological staining technique methods are adaptable to the determination of the origin of fibrin in or on the vessel wall If the method is applicable it would surely be valuable to validate or deny Duguid s concept Then too there is the perennial question of the origin and composition of fibrinoid so much in the fore ground recently due to Dr Lewis Thomas experiments

Dr Gitlin One of Dr Duguid s former students Dr Charles Levine has been interested in the problem and is now setting up to study it

Chairman Hartroft I think it should be done in more than one laboratory since it is so important

Dr Gitlin It s all at Harvard

Dr Shainoff The fluorescent labeled antibody technique does not differentiate between fibrinogen and fibrin A number of hypotheses can be offered for the deposition of fibrinogen one of which is that it forms an insoluble complex with mucopolysaccharides The labeled antibody technique in itself would probably not be sufficient for evaluation of Dr Duguid s hypothesis

Dr Gitlin This is true But the precipitation of fibrinogen under *in vivo* con ditions by other substances as suggested by Dr Lewis Thomas seemed unreason able to us Dr Thomas is now restudying this question with the electron micro scope Perhaps one of the electron microscopists can answer this better than I He now concludes this is indeed fibrin unless I am misquoting him

Dr Shainoff The hypothesis as Dr Astrup presented it is that fibrin is being produced by thrombin Microscopic methods cannot tell us how and when the clots appear Other proteolytic enzymes can produce clots with regular perio dicity as seen by the electron microscope It is also possible that fibrin clots are produced subsequent to the deposition of fibrinogen

Dr Gitlin But the ultimate results are still a derivative of fibrinogen

Chairman Hartroft Dr Gitlin would you like to say loudly and clearly that fibrinoid is derived from fibrin?

Dr Gitlin I wouldn t say that I would simply say that fibrinoid contains fibrin or more properly a derivative of fibrinogen I believe the bulk of fibrinoid is fibrin The question has come up as to whether or not fibrinoid might also not be an antigen antibody precipitate and I have a slide here which shows fibrinoid *necrosis in a patient with agammaglobulinemia with* no gamma globulin to be found anywhere and the fibrin deposit within the areas of fibrinoid

Dr Sherry I would like to ask Dr Gitlin about the absolute specificity of the method The best preparations of fibrinogen still contain about 1% contamination with other plasma proteins If this impure fibrinogen is converted to fibrin by the addition of thrombin then another contaminant is added How then is one sure that the antiserum is specifically directed against fibrin?

Dr Gitlin I think this is one of the most important questions one could ask about this particular procedure You don t have to restrict it to fibrin Unless you

just described for the β lipoproteins Since the lipoproteins differ in their cholesterol content as well as in other characteristics it is necessary to specify precisely what we have in mind when we speak of "cholesterol The metabolism of the β lipoproteins as we envisage it is illustrated in Fig 11

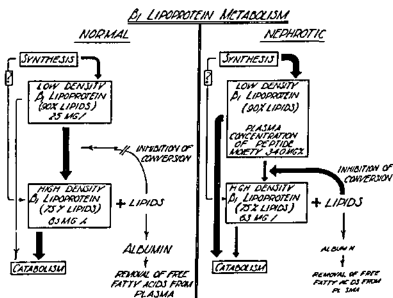


FIG 11 A simplified scheme for β lipoprotein metabolism In the normal person the low density lipoproteins are converted to S_f 3 to 9 lipoproteins by means of lipoprotein lipase and the unesterified fatty acids resulting therefrom are bound by albumin In the nephrotic individual with elevated S_f 20 to 400 lipoproteins synthesis of these lipoproteins is increased and the major pathway for elimination is through direct catabolism with some passing through S_f 3 to 9 lipoproteins In these individuals conversion of low density to high density lipoproteins is inhibited presumably through the absence of albumin for binding unesterified fatty acids and other lipids which can inhibit the conversion through depressed amounts of lipoprotein lipase and through the presence of other inhibitions of lipoprotein lipase The concentrations of lipoprotein given are in terms of the peptide moiety only and represent the averages of the concentrations in the group of children studied (Reprinted with permission from *Pediatrics* 19 657 1957)

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DR VON KAULLA What do you think is the role of the lymph in the reappearance of the antibodies? Also do you have experience with the hyaline membranes formed in guinea pigs exposed to oxygen and do you know whether they contain fibrin? A material described as fibrin has been found in the hyaline membrane

DR. GITLIN Yes it does contain fibrin I would not want to say however that high oxygen is the cause of hyaline membrane disease in children

DR VON KAULLA But in guinea pigs you have no free thromboplastic material in the alveoli

DR. GITLIN But you do because oxygen is toxic to the pulmonary capillaries and tissue damage occurs Otherwise an effusion would not have appeared As to the role of the lymphatics I did not show the extravascular circulation that exists for plasma protein but there is a diffusion of protein across the capillary wall and the bulk of this is then transported by the lymphatics back into the circulation The concentration gradient is always from the capillary to the interstitial fluid

DR. VON KAULLA We were interested in how quickly and at what concentration radioactive material injected intravenously appears in ovarian follicular fluid. If we injected I^{131} intravenously in woman we demonstrated in less than 1 hour at least half the concentration of the material in the blood appears in follicular fluid

DR GITLIN Yes About half the plasma protein in the vascular system diffuses out every day in man

DR WHITE As you probably know Dr Howard Eder has shown this transfer of lipid from one protein to another *in vitro* These data of yours *in vivo* are extremely interesting To what extent is this a normal phenomenon?

DR. GITLIN These proteins were deliberately labeled in the peptide moiety so we know nothing about lipid transfer other than that there has been a decrease in the density meaning that one lipoprotein was converted to another lipoprotein by loss of lipid

DR. WHITE With double labeling could one do this?

DR GITLIN Yes

DR WHITE When the low density lipoproteins build up to pathological levels what has happened to the normal mechanisms concerned with this transfer?

DR GITLIN They are blocked We have such an example in the nephrotic syndrome There are two theories not mutually exclusive One is that there is an inhibitor to the conversion and the other is a deficiency of lipoprotein lipase We incline toward the latter but either or both can be operative

DR WHITE In the nephrotic there might also be a protein manufacturing deficiency

DR GITLIN Or loss not deficiency in synthesis

DR WINTER Are these proteins which are interconverted antigenically the same?

DR GITLIN No the protein moieties are apparently immunologically similar but not identical

DR ADLERSBERG We have tried to correlate histochemical and chemical techniques in regard to the possible relationship between lipid deposition in arterial walls and the mucopolysaccharide matrix which Dr Moon mentioned earlier

Our studies were performed in cholesterol fed rabbits in which experimental xanthomatosis was produced In the skin of a normal rabbit stained with colloidal iron the typical blue reaction is seen only around the hair follicles

In a hematoxylin-eosin stain of a rabbit treated approximately 1 month with 1 gm of cholesterol daily foam cells are seen in small quantities in the corium But if colloidal iron is used one sees in comparison with the one before the tre

study the antiserum from which you prepare the fluorescent antibody you might just as well not label it antiserum

In this particular instance we used purified fibrinogen the best preparation was about 95% clottable fibrinogen We clotted this with bovine thrombin and washed the fibrin many times to prevent occlusion Antiserum was made against this The antibody was purified by adsorption of the antiserum with human serum We then tested the antibody by means of the quantitative precipitin reaction against purified fibrinogen and by agar diffusion we could find only one reacting component and that was the fibrinogen

DR WHITE *How do you solubilize the fibrin for administration?*

DR GITLIN We dont It was injected through a No 18 needle in suspension with aluminum hydroxide gel subcutaneously

CHAIRMAN HARTROFT Would it be good to test the specificity of the method by reaction of the antiserum with fibrinogen prior to its application to the tissue? This treatment should block the staining reaction

DR GITLIN Yes and no The trouble with this method is that if one does not prepare the controls properly one can get any answer one wants For this reason we have not applied the method extensively We prefer to use those systems we understand

For example if the section is overlaid with unlabeled antiserum and then stained with labeled antiserum this is not a complete control since the antigen antibody reaction is reversible You may have eliminated only one part of the re action The process of adsorbing the antiserum with specific antigen is not very good because what the original antigen is is problematical unle s the antiserum has been studied If the labeled antiserum can be absorbed with specific antigen or thrown into the zone of the antigen excess a complete block in reaction can be shown But the most important question remains as to what antibodies were really in the antiserum

DR SHAINOFF How do you know the fibrin did not appear post mortem?

DR GITLIN Many of our tissues were biopsies The myocardial pictures were postmortem

DR GROSS I would like to raise one other question concerning quantitation With this beautiful almost homogeneous staining is it true that one still cannot tell whether there is 1 50 or 95% fibrin?

DR GITLIN Absolutely

DR GROSS One cannot say that fibrinoid is fibrin or that a lesion is fibrin because it gets homogeneously stained?

DR GITLIN *I did not say that I said it contains it*

CHAIRMAN HARTROFT Cant one do a dilution with the labeled material?

DR GITLIN This is a titration in the test tube that was abandoned about 20 years ago This type of titration is difficult to interpret In order to get a really quantitative result by diluting antiserum one must also have an antigen dilution as well The only way one can tell whether it is 1 50 or 100% is to label the anti serum Having a highly specific antiserum one could label it with a radioactive substance such as iodine After determining the specific activity of the specific antibody stain the sections with this and then count them

CHAIRMAN HARTROFT We had hoped to apply this to quantitative measure ment as well as to labeling

DR GITLIN One can also do it by elution of the antibody from the section and measurement of the amount of fluorescence in the solutions The radioactivity has an advantage in that the fluorescence can be quenched

AUTHOR INDEX

Numbers in parentheses are reference numbers and are included to assist in locating the reference where the authors names are not mentioned on the page Numbers in italics refer to the page on which the reference is listed at the end of the article

mendous accumulation of acid mucopolysaccharides in the corium We thought that this was the matrix of acid mucopolysaccharides in which lipid is being de posited and that this illustrated the alleged relationship between the two com pounds In the skin of a rabbit fed cholesterol for 10 months massive accumulation of lipid and crystals in the corium is seen

A colloidal iron stain shows there is much less blue discoloration than after 1 month of cholesterol feeding Apparently once the lipid is deposited there is much less mucopolysaccharide in the tissue But other possibilities would be changes in staining qualities and perhaps also that the large quantities of lipid displaced the mucopolysaccharides from the tissue The histochemical changes in the aorta of cholesterol fed rabbit resemble the findings in the ground substance of the skin

The following data obtained in the aortas of these animals came from a co operative study with Dr A J Bollet in Detroit After periods of 1 to 7 months of cholesterol feeding the aortas were removed dried and defatted extracted with 0 5 *N* NaOH and the acid mucopolysaccharide isolated after removal of protein The uronic acid content of the mucopolysaccharide was determined by the carbazole and orcinol methods before and after digestion with testicular hyaluronidase

Aortas of normal rabbits contained 341 ± 3 9 mg of uronic acid in muco polysaccharide per 100 gm of dried fat free tissue by the carbazole method the carbazole orcinol ratio was 1 5 After hyaluronidase incubation the concentration averaged 175 ± 10 4 mg % (carbazole) No changes in these levels were noted in rabbits fed cholesterol for 34 62 and 100 days despite progressive increase in serum lipids and development of 1 to 2 plus atherosclerosis the corresponding concentrations of the aortas were 342 312 and 341 mg % (carbazole) Only in rabbits fed cholesterol for 186 days and more was there a decided increase in the mucopolysaccharide content of the aorta to an average of 512 ± 36 mg % by the carbazole method ($p \cong 0\ 01$) No change occurred in the carbazole orcinol ratio (1 3) or in the amount of mucopolysaccharide after hyaluronidase digestion

These observations indicate that an increase in the mucopolysaccharide concen tration of the aorta occurs in the cholesterol fed rabbit but only after prolonged feeding with high serum lipid levels maintained over several months and with the development of moderate to severe atherosclerosis Caution is advisable in the in terpretation of histochemical findings concerning the connective tissue ground substance during atherogenesis

Dr Meyer I do not know anything about the reliability of the methods The chemical method of Bollet is not sufficient for the characterization of either the quantity of polysaccharides or certainly not for the type of polysaccharides

Dr Adlersberg What was determined was the uronic acid in mucopolysac charide The technique will be described in detail by Dr Bollet

Chairman Hartroft I think it is interesting to see tissues other than the vessel being studied in this type of rabbit

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